THE DIFFERENTIAL TOXICITY OF AMMONIUM SALTS

By KENNETH S WARREN

(From the United States Department of Health, Education, and Welfare, Public Health Service, National Institutes of Health, National Institute of Allergy and Infectious Diseases,[1] Bethesda, Md.)

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The relationship between liver disease and ammonia toxicity has been known since the discovery of meat intoxication in Eck fistula dogs (1–4). The similarity between this syndrome and clinical hepatic coma stimulated the study of ammonia toxicity in humans. This proceeded from oral administration (5–10) to intravenous infusion (11, 12) and finally to the development of an "ammonia tolerance test" (13, 14). Ammonia toxicity studies have been performed in animals by the intravenous (15–24), oral, subcutaneous, and intraperitoneal routes (25–29).

Many different ammonium compounds were used in the above studies with the tacit assumption that their toxicities were similar. In order to test that assumption, intravenous LD50's were determined in mice under controlled conditions.

MATERIALS AND METHODS

Intravenous LD50's were determined in young Swiss albino mice, weighing 18 to 22 Gm. (average, 19.7 Gm.). One ml. of isotonic saline containing the ammonium compounds (reagent grade) in varying concentrations was injected into a tail vein during an average time of 1.9 seconds. The lethal effect was determined in 10 animals for each of three to five concentration levels. The mice were observed continually until all signs of acute toxicity disappeared and then were examined during the course of several days for the appearance of any latent toxicity.

Blood pH was determined with a Beckman Model GS meter, equipped with a 290–80 blood electrode, on heart blood obtained from live, unanesthetized animals. The effect of each ammonium salt on the blood pH was measured within one minute of its intravenous administration.

The LD50's and approximate 95 per cent confidence limits were obtained by the Carver method (30).

RESULTS

When the intravenous LD50's of ammonium chloride, citrate, acetate, bicarbonate, carbonate, and hydroxide were determined, differences in their toxicities were noted. On studying the relationship of the LD50's to the pH's of the compounds in isotonic saline and their effect on the blood pH (normal, 7.46 ± 0.02), it was observed that the toxicity of the compounds increased with a rise in pH, as shown in Figure 1. A notable exception was ammonium citrate which, although it had the lowest pH, displayed the highest toxicity. The data pertaining to this seemingly aberrant effect are not represented in Figure 1 but will be presented below, as the toxicity of ammonium citrate does not appear to be due to the ammonium moiety.

When the ammonium salts, with the exception of citrate, were administered in a single rapid intravenous dose (between the minimal and maximal lethal doses), a reaction began in 2 to 5 seconds, characterized by hyperventilation and clonic convusions. This was followed by either a fatal tonic extensor convulsion or the gradual onset of coma over the course of three to five minutes. The mice remained in a comatose state for approximately 30 to 45 minutes, showing no response to touch or light, but moving convulsively to sound stimuli. During this time a tonic convulsion and death, without cardiac arrest, could occur at any time, but those mice which survived made a rapid and complete recovery.

There was no discernible effect from the injection of 1 or even 2 ml. of isotonic saline. The injection of the maximum lethal dose of ammonium chloride over increasingly prolonged time periods of one-half, one, two, four, and six minutes revealed only a slight diminution of toxicity at four to six minutes. Since the toxicity of the ammonium salts appeared to vary with the pH, the effects of $Na_2CO_3/NaHCO_3$ and Na_2HPO_4/NaH_2PO_4 buffers with concentrations and pH's approximating the maximum lethal doses of ammonium chloride and ammonium hydroxide were studied. There was no evidence of toxicity. In

[1] Laboratory of Tropical Diseases.

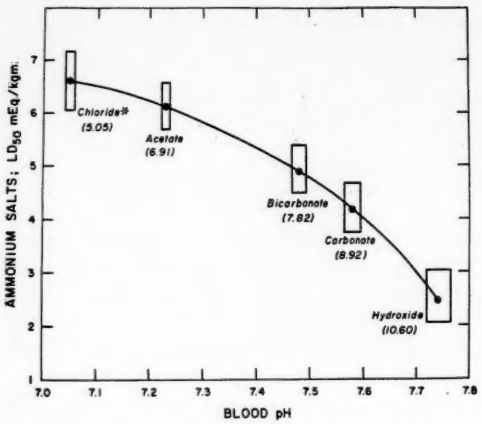

FIG. 1. LD50's OF AMMONIUM SALTS AND THE RESULTANT pH OF BLOOD ONE MINUTE AFTER INJECTION

The rectangle surrounding the points determined denotes, on the ordinate, the approximate 95 per cent confidence limits, and on the abscissa, the standard error of the mean pH—average for 10 animals.

* pH of the solution administered.

order to determine the effect of the anionic component of the ammonium salts, sodium salts of each compound were injected in a concentration equivalent to the maximum lethal dose, and again no toxicity was noted. Attempts to achieve a prior alkalosis, either metabolic or respiratory, were negated by the overpowering effect of the intravenously administered ammonium compounds.

Since the physical state in which ammonia exists might influence its toxicity, it was of interest to calculate the relative amounts of ammonium ion and ammonia gas at the pH's of the LD50 concentrations. In order to do this the following formula was derived from the mass action law:

$$\frac{X}{(Y - X)(H^+)} = 1.8 \times 10^9,^2$$

where X equals NH_4^+ and Y equals total ammonia. The data calculated from the above formula, summarized in Table I, reveal that as the blood pH rises, the amount of ammonia gas relative to ammonium ion increases markedly.

TABLE I

The effect of blood pH on the ratio of ammonia gas to ammonium ion

Ammonium salt	LD_{50} concentration *mEq./L.**	Blood pH	NH_3 *mEq./L.*	NH_4^+ *mEq./L.*	Ratio NH_3/NH_4^+
Chloride	135.0	7.05	0.84	134.16	0.0063
Acetate	124.6	7.23	1.16	123.44	0.0094
Bicarbonate	100.9	7.48	1.67	99.23	0.0168
Carbonate	89.3	7.58	1.85	87.45	0.0212
Hydroxide	50.5	7.74	1.50	49.00	0.0306

* One ml. administered intravenously to each animal.

TABLE II

The toxicity of ammonium citrate

Solution administered	LD_{50} concentration of citrate salts *mEq./Kg.*	Solution pH
Ammonium citrate	1.58 (1.52–1.63)*	4.86
Ammonium citrate + calcium gluconate	8.40 (8.03–8.79)	4.63
Sodium citrate	1.94 (1.86–2.02)	4.90†

* Approximate 95 per cent confidence limits.
† pH adjusted to 4.90 with HCl.

When ammonium citrate was administered, toxicity appeared to develop in a manner somewhat different from that of the other ammonium compounds. The animals immediately began to leap about exhibiting tetanic movements of all limbs, followed by the sudden onset of apnea and death, with the heart still beating. They never developed the clonic-tonic convulsions typical of ammonia toxicity, and either died in a few minutes or recovered rapidly, with no subsequent development of coma. In order to test whether this effect was due to the citrate moiety and the induction of hypocalcemia, enough calcium in the form of calcium gluconate was added to ammonium citrate, on an equivalent weight basis to bind the citrate completely. As is shown in Table II, the toxicity was reduced below that of ammonium chloride. The toxicity of sodium citrate at a similar pH was approximately the same as that of ammonium citrate. Calcium gluconate had no effect on ammonium chloride toxicity.

DISCUSSION

The material presented in Table I, illustrating an increased ratio of ammonia gas to ammonium ion with a rise in blood pH, may explain the differential toxicity of the ammonium salts studied.

[2] This figure was derived from the dissociation constant of NH_4OH.

Preliminary data gathered in this laboratory (31) revealed that the greater part of the ammonia administered in a single injection passed from the blood in less than three minutes. In spite of this fall in ammonia level, our mice did not become comatose for 3 to 4 minutes and they remained in coma for at least 30 minutes. This may indicate a rapid and early passage of ammonia into the brain. Therefore, the effect of pH on the blood-brain-barrier may be related to either the greater permeability of free than ionized ammonia, or to an effect of pH directly on the barrier itself.

The pH of the medium in which ammonium salts are dissolved has been shown to influence the ability of ammonia to cross cell boundaries in both red blood cells (32) and other tissues (33). This appears to be due to the amount of ammonia gas versus ammonium ion in that the unionized ammonia has a much greater ability to penetrate cells. Jacobs and Stewart (33) have shown that the ammonium salts of weak acids, such as acetate, bicarbonate, and benzoate, penetrate all cells with great ease, while ammonium salts of strong acids, chloride, nitrate, and sulfate apparently do so no more readily than the corresponding potassium and sodium salts. The latter salts have been shown to cross the blood-brain-barrier at an extremely slow rate (34), requiring a much greater time to equilibrate with the brain than with other tissues. In all probability, ammonia gas can penetrate this barrier as rapidly as other gases, and as readily as it passes other tissue barriers.

A direct effect of pH changes on the blood-brain-barrier has been reported by Broman and Lindberg-Broman (35) who found that extreme changes in pH increase the permeability of the blood-brain-barrier to trypan blue. Fisher, Troast, Waterhouse, and Shannon (36) have shown that the amount of a given sulfa drug that crosses the barrier is dependent on the pK of the compound; the higher the pK the greater the passage. The question resolves itself, therefore, as to whether more ammonia crosses the barrier at high pH's because of the increased ammonia gas to ammonium ion ratio, or whether there is a direct effect on the blood-brain-barrier itself, or both. Animal studies now in progress may provide an answer.

Other investigators have studied the toxicity of different ammonium salts. As early as 1893, Marfori (15) noted that ammonium carbonate was much more toxic than either ammonium lactate or tartrate during slow intravenous infusion into rabbits and dogs. Roberts, Thompson, Poppell, and Vanamee (23) have recently speculated that an elevation of blood pH could enhance the toxicity of ammonia; nevertheless, several investigators have found no such relationship. Following the administration of ammonium compounds to dogs, Karr and Hendricks (20) felt that the effect of pH on ammonia within physiological limits was negligible, and although Iwahashi (29) noted differences in toxicity of the compounds in mice, he could find no consistent pattern. Eiseman, Fowler, White, and Clark (24) administered ammonium salts intravenously to dogs and concluded that there was no relationship between pH and ammonia toxicity. Since

TABLE III

Tabulation of the data of Eiseman and co-workers (24) on the infusion of ammonium compounds into dogs

Ammonium salt	Concentration and rate of infusion	Total time infused *hours*	Highest blood ammonia level *μg. NH_3/ml.*	Blood ammonia during coma *μg. NH_3/ml.*	Total NH_3 infused *Gm.*	Blood pH at coma	Remarks
Chloride	5% 2 ml./min. 2 hrs. 1 ml./min. 2 hrs.	4	175	90	5.73	6.9	Convulsion at 4 hrs.
Hydroxide and HCl, pH 8.8	1% 1 ml./min.	5	40	35	1.46	7.4	
Hydroxide	1% 1 ml./min.	4.5	50	20	1.31	7.6	
Citrate	4% 2 ml./min.	3	30	20	2.14	7.2	Convulsion, 2 hrs.; tremors, 40 min.

their data are particularly pertinent to this discussion, they are summarized in Table III. The concentrations of the drugs administered, as well as the rates and times of infusion and the blood ammonia levels at onset of coma, were not uniform. The symptomatology evinced by the dog given ammonium citrate did differ from those given the other salts in that there were tremors at an early stage and a convulsion about midway in the course of the infusion. Their data not only suggest a relationship between blood pH and ammonia toxicity, but also reveal the excessive toxicity of ammonium citrate.

Since ammonium citrate is being used with increasing frequency in both animal and human studies (12, 14, 24), it is important to note that in a single intravenous lethal dose in mice the citrate moiety is more toxic than the ammonium moiety. The mice die in tetany due to hypocalcemia rather than in a manner consistent with ammonia toxicity. Therefore, the use of ammonium citrate in human experimentation, particularly when administered via the intravenous route, might be contraindicated.

In relation to clinical hepatic coma and its therapy, Gyorgyi and Kleinschmidt (37) in 1927 found that dogs with Eck fistulas in meat intoxication were alkalotic. Vanamee, Poppell, Glicksman, Randall, and Roberts (38) showed, and it was confirmed by Robin, Whaley, Crump, and Travis (39), that the majority of patients in hepatic coma develop a respiratory alkalosis due to hyperventilation with blood pH's as high as 7.70. This alkalosis may effect significantly the toxicity of a given amount of ammonia in the blood and may be one of the factors accounting for the poor relationship between blood ammonia levels (arterial and venous) and clinical hepatic coma.

Gyorgyi and Kleinschmidt (37) noted further that the oral administration of hydrochloric acid brought about a rapid improvement even in severely intoxicated animals. The administration of acidifying agents or carbon dioxide to patients in hepatic coma may therefore prove to be beneficial.

SUMMARY

1. Intravenous LD50's of several ammonium salts were obtained in mice.

2. The ammonia toxicity of the chloride, acetate, bicarbonate, carbonate, and hydroxide salts increased in relation to their effect in raising blood pH.

3. This change appeared to be related either to the effect of pH on the NH_3/NH_4^+ ratio and the ability of ammonia gas to cross the blood-brain-barrier, or to a direct effect of pH on the barrier.

4. The toxicity of ammonium citrate, under the above conditions, does not appear to be related to ammonium toxicity, but to hypocalcemic tetany induced by chelation of calcium by the citrate moiety.

5. Evidence was cited from the literature supporting these results obtained in mice by similar experiments in rabbits and dogs.

6. Therapeutic implications are discussed in the light of the recent findings that respiratory alkalosis is a frequent accompaniment of the hepatic coma syndrome.

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A CORRELATION OF CLINICAL AND HEMODYNAMIC STUDIES IN PATIENTS WITH HYPOTHYROIDISM [1]

By JOHN S. GRAETTINGER, JOSEPH J. MUENSTER,[2] CARLO S. CHECCHIA,[3] ROBERT L. GRISSOM,[4] AND JAMES A. CAMPBELL

(From the Cardio-Respiratory Division, Department of Medicine, Presbyterian Hospital, and Department of Medicine, University of Illinois College of Medicine, Chicago, Ill.)

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Congestive heart failure has for many years been considered to be a possible complication of myxedema and, although heart disease of other etiologies has been described in patients with hypothyroidism, myxedematous involvement of the myocardium has sometimes been assumed to be a cause of congestive heart failure (1–4). More commonly the term "myxedema heart" is used with the connotation of actual or potential myocardial malfunction associated with the myxedematous state (5–10). Postmortem studies have demonstrated that hydropic vacuolization of the myocardium may occasionally be found in the rare patient who succumbs with myxedema (11–14), but clinical data which support the concept of involvement of the heart to the extent of impairing cardiac function are not conclusive. Indeed, the improvement in cardiac decompensation which commonly occurs when a patient with severe heart failure is rendered hypothyroid is well documented (15, 16). Physiologic studies in patients with hypothyroidsm have established that the cardiac output is low and that the circulation time is often moderately prolonged. In the majority of patients studied the reduction in cardiac output has been found to be proportional to the decreased oxygen consumption with the arteriovenous difference normal; other investigators have noted a somewhat greater reduction in cardiac output than in oxygen consumption with a somewhat increased arteriovenous difference (17–29). Pressures within the heart and the pulmonary and systemic circulations have been found to be within normal limits (22, 26). These data obtained in resting patients suggest that although the cardiac output is low in hypothyroidism it is adequate in the presence of decreased circulatory load. Since symptoms and hemodynamic abnormalities frequently occur only during exercise in patients with marginal cardiac compensation, the finding of normal values at rest in patients with hypothyroidism does not necessarily exclude impaired myocardial reserve. Normal circulatory responses to exercise were noted in two patients studied by Ellis, Mebane, Maresh, Hultgren, and Bloomfield (22). In the present paper we are reporting a correlation of the clinical syndrome of hypothyroidism with physiologic data obtained at rest and during exercise in twelve patients studied as part of an investigation of the relationship of various types of clinical heart disease and congestive failure to hemodynamic changes. These patients will be compared with healthy subjects and with patients who were in congestive heart failure from ultimately fatal myocardial disease (30).

MATERIAL AND METHODS

Twelve patients with hypothyroidism have been studied whose ages ranged from 17 to 59 years; 10 were over 40 and 4 over 50. All had had symptoms of hypothyroidism for over one year. Dry skin and hair, puffiness of the face and other signs of myxedema were present in each at the time of study. Confirmatory laboratory data are shown in Table I. None had distended neck veins or persistent signs of pulmonary congestion, although transient rales were present at the lung bases in one patient (R. C.). Another patient (E. D.) had rheumatic heart disease with mitral insufficiency and stenosis with characteristic auscultatory and roentgenographic findings. Slight hepatomegaly was present in five patients, pitting edema of the legs in four, and ascites in one. Enlargement of the cardiac silhouette was evident in the teleroentgenograms of eight patients. The contour in three was regarded as diagnostic of pericardial effusion and of right and left ventricular and left atrial enlargement in the patient with rheumatic heart disease. Generalized enlargement without

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[3] Present address: Cleveland Clinic, Cleveland, Ohio.

[4] This work was done during the tenure of a John and Mary R. Markle Scholarship in Medical Science. Present address: Department of Medicine, University of Nebraska, Omaha, Nebraska.

diagnostic configuration was noted in the films of the other four patients. Arm to tongue circulation time (Decholin®) was normal in three (R. B., 17 seconds; V. B., 17 seconds; C. R., 15 seconds) and prolonged in three (L. L., 25 seconds; A. S., 24 seconds; R. C., 22 seconds) of the six patients in whom this determination was made; in one of the patients (L. L.) with prolonged circulation time, the heart was enlarged and a small pleural effusion was present. Total blood volumes were normal to low in the five patients in whom it was measured: E. C., −19 per cent; L. L., +7 per cent; C. R., −33 per cent; R. B., −17 per cent; E. L., −33 per cent. The electrocardiograms of each of these patients revealed normal sinus rhythm; one showed normal voltage of QRS-T, five low voltage of the QRS-T deflections, and six low voltage of the T waves. The electrocardiogram of E. D. with rheumatic heart disease also suggested right ventricular hypertrophy. Their hemoglobins ranged from 7.6 to 11.8 Gm.

Data obtained in seven patients with typical clinical congestive failure from ultimately fatal myocardial disease without evidence of valvular heart disease are compared with the data obtained in the patients with hypothyroidism. Postmortem examination disclosed nonspecific myocarditis in four patients, amyloid disease involving the myocardium in another, and myocardial atrophy following pericardial resection in another. No postmortem examination was obtained in the seventh patient who clinically was diagnosed as myocardial disease of unknown etiology.

The studies were performed in an air-conditioned room on patients in the postabsorptive state, usually after a sedative dose of sodium allyl barbiturate. Cardiac catheterization was performed in the usual manner after insertion of a Cournand needle into a brachial artery. Simultaneous venous blood samples from the pulmonary artery (nine patients) or right atrium (three patients) and arterial samples were collected anaerobically together with expired air samples in Douglas bags at rest and during the fourth minute of exercise on a stationary bicycle with the patient in the supine position. Arterial and venous oxygen contents and arterial oxygen capacity were determined by the method of Van Slyke and Neill (31). Oxygen and carbon dioxide concentrations in expired air were measured either by the micro-Scholander technique (32) or by the use of a Pauling oxygen analyzer and Haldane manometric apparatus. Intravascular pressures were recorded from strain gauges placed 10 cm. above the level of the patient's back, and mean pressures were obtained by planimetry. I^{131} was used for determination of blood volume. Body surface area was determined on the basis of height and weight at the time of study in all patients.

RESULTS

The hemodynamic data together with the hemoglobin and metabolic rate at the time of study and the serum cholesterol or I^{131} uptake of each patient are presented in Table I. The mean data and standard errors obtained in five healthy subjects are tabulated at the bottom of each column together with the probability of the observed differences between the hypothyroid patients and healthy subjects being attributable to chance.

Cardiac output index

The resting cardiac index of each patient was considerably below normal without apparent relationship to the hemoglobin level, heart size or presence or absence of pericardial effusion, and the difference of the mean of 1.88 ± S. D. 0.8 L. per minute per M.² from the normal was highly significant. During exercise, an increase in cardiac output occurred in all patients; in two, E. D. with rheumatic heart disease and E. L. who clinically presented the most severe hypothyroidism in this group of patients, the increase was slight. The mean increase in output for the entire group to 3.05 ± S. D. 0.77 L. per minute per M.² (+62 per cent) was highly significant and represents a greater proportional increase than has usually been observed in healthy persons performing the exercise test (+51 per cent).

Cardiac output:oxygen consumption ratio

The ratio of cardiac output to oxygen consumption (the reciprocal of the arteriovenous difference) in the hypothyroid patients was not significantly different from normal either at rest or exercise although it was below the normal range for our laboratory of 2.32 ± S. D. 0.13 in four patients. These relationships are plotted in Figure 1 together with the regression line of these data measured at rest. The cardiac output at rest in these patients with myxedema was directly related to the level of oxygen consumption ($r = 0.71$, $p < 0.01$). The ratio of the increase in cardiac output to the increase in oxygen consumption during exercise appears in Table I and is represented by the slope of the lines connecting the rest and exercise data for each patient in the figure. With one exception, E. D. with mitral stenosis and insufficiency, this ratio was as great or greater than that usually found in healthy persons (33).

Stroke volume index and heart rate

The considerable decrease in basal cardiac output in these patients was primarily attributable to

TABLE I

Hemodynamic data in patients with hypothyroidism compared with those of normal subjects

Patient	Age—Sex	Surface area *M.*²	Serum cholesterol	Basal metabolic rate %	Hemoglobin	Oxygen consumption *ml./min./M.*² R*	Oxygen consumption *ml./min./M.*² E*	Cardiac output index *L./min./M.*² R	Cardiac output index *L./min./M.*² E	Cardiac output *L./min.* × 100 / O_2 consumption *ml./min.* R	Cardiac output *L./min.* × 100 / O_2 consumption *ml./min.* E	Δ Cardiac output (*ml.*) / Δ 100 *ml.* O_2 consumption
A. R.†	56—M	1.60	374	−16	10.9	96	331	2.09	3.95	2.18	1.19	791
E. C.‡	41—F	1.06	505	−19§	8.6	101	142	2.19	3.32	2.17	2.34	2,756
I. C.†	44—F	1.66	480	−20§	10.6	98	173	2.15	4.15	2.19	2.40	2,667
R. C.‡	52—M	2.22	268	−21§	11.4	100	230	1.97	3.34	1.97	1.45	1,015
L. L.†	59—M	1.83	395	−23§	10.9	96	235	1.64	3.23	1.71	1.37	1,144
C. R.	17—F	1.55	345	−30	8.5	90	208	2.18	3.27	2.42	1.57	924
V. B.	57—M	1.70		−32§	10.9	85	146	1.74	2.56	2.05	1.75	1,344
A. S.	43—F	1.66	670	−35	9.2	78	228	1.89	3.71	2.42	1.63	1,213
E. D.†,‖	41—F	1.53	420	−37	10.9	77	129	1.62	1.86	2.10	1.44	462
R. B.	46—M	1.86	504	−37	11.8	79	179	1.87	2.76	2.37	1.54	890
J. P.‡	36—F	1.76		−38	7.6	76	152	1.84	2.81	2.42	1.85	1,276
E. L.†	43—F	1.86	570	−46	10.4	67	97	1.35	1.63	2.01	1.68	933
Mean						87	188¶	1.88	3.05¶	2.18	1.68¶	1,285
Standard error						3	18	0.07	0.22	0.06	0.12	204
Normal subjects—5												
Mean						126	206¶	2.92	4.42**	2.32	2.22	2,984
Standard error						5	11	0.10	0.54	0.06	0.39	1,123
p						<0.01	>0.5	<0.01	<0.02	<0.3	<0.1	<0.05

* R = Rest. E = Exercise.
† Enlarged cardiac silhouette.
‡ Pericardial effusion.
§ I^{131} uptake < 10 per cent.

a significant decrease in stroke volume in the presence of a low normal heart rate. During exercise, the rise in output observed occurred in the majority of the patients primarily by an increase in stroke volume, but in two patients (C. R. and R. B.) the increase was primarily because of an increase in heart rate. The two patients who increased their cardiac outputs only slightly during exercise also failed to increase their pulse rates significantly. For the group, the increase in both heart rate and stroke volume index was significant.

Arteriovenous oxygen difference

The arteriovenous oxygen difference in 4 of the 12 patients was higher than the normal range in our laboratory (4.33 ± S. D. 0.24 volume per cent), but the mean value for the group, 4.66 ± S. D. 0.52, although in the high normal range, did not differ significantly from the normal. During exercise, the mean arteriovenous difference increased to 6.15 ± S. D. 0.19 which was not significantly higher than in healthy persons. The arteriovenous differences observed in the two patients who demonstrated only slight increases in cardiac output (E. D. and E. L.) did not differ from those of the other patients. The mean oxygen extraction ratios (arteriovenous difference divided by arterial oxygen content) of 36.7 ± S. D. 4.4 per cent at rest and 46.9 ± S. D. 7.2 per cent at exercise were not significantly higher than these observed in euthyroid patients with similar levels of anemia (30).

Right atrial pressure

The mean atrial pressures were slightly elevated at rest in five patients (A. R., I. C., R. C., E. D., and J. P.) but did not increase significantly with exercise. In two patients (I. C. and E. L.) atrial pressure rose slightly with exercise. In none of the patients with normal size of the cardiac silhouette was elevation of the atrial pressure at rest or exercise observed. The mean for the group did not differ significantly from the normal either at rest or during exercise.

Right ventricular pressure

Right ventricular pressure tracings were recorded in eight patients. Tracings were obtained in two of the four patients with normal sized cardiac silhouettes (A. S. and C. R.). The pressure dropped to zero after systole and rose to 5 mm. Hg at the end of diastole. Pressure curves were recorded in each of the three patients with definite pericardial effusions and "diastolic dips"

TABLE I—*Continued*

Hemodynamic data in patients with hypothyroidism compared with those of normal subjects

Stroke volume index *ml./beat/M.²*		Heart rate *beats/minute*		A-V oxygen difference *ml./100 ml.*		Right atrial pressure *mm. Hg*		Mean pulmonary artery pressure *mm. Hg*		Mean brachial artery pressure *mm. Hg*		Total pulmonary resistance *dynes cm. sec.⁻⁵*		Total systemic resistance *dynes cm. sec.⁻⁵*	
R	E	R	E	R	E	R	E	R	E	R	E	R	E	R	E
30	37	70	108	4.61	8.38	8	10	15	30	108	119	359	379	2,584	1,505
28	36	79	93	4.58	4.28	5		18	22	120	120	617	755	4,116	2,724
30	53	72	79	4.58	4.17	11	14	23	26	106	120	515	302	2,372	1,395
38	48	52	70	5.09	6.90	13	13	22	23	70	76	401	248	1,700	1,342
28	45	57	70	5.90	7.28	5	7			105	117			2,815	1,582
32	33	68	99	4.11	6.39	2	3	7	10	94	102	166	158	2,222	1,607
37	51	47	50	4.88	5.71	1		8	13	92	104	225	238	2,492	1,906
26	44	74	85	4.16	6.13		2	8	11	102	100	204	143	2,604	1,297
26	31	62	60	4.75	6.83	7	7	20	26	71	75	645	732	2,288	2,110
37	36	51	76	4.21	6.43	2	3			100	117			2,290	1,822
28	36	65	79	4.10	5.42	10	6	30/6‡‡		84	84			2,072	1,239
19	22	71	73	4.95	5.94	5	9	18	23	106	103	573	603	3,375	2,698
30	39¶	64	79¶	4.66	6.15¶	6	7	15	20¶	97	103¶	412	395	2,577	1,733¶
0.5	0.5	3	5	0.15	0.34	1	1	2	2	4	5	62	80	183	160
41	51	72	86**	4.33	5.01	3	3	15	20**	94	99	260	258	1,558	1,124
2	6	3	3	0.11	0.76	0.5	1	2	3	7	3	49	50	161	125
<0.01	<0.1	<0.2	<0.4	<0.2	<0.2	<0.3	<0.1	>0.5	>0.5	>0.5	>0.5	<0.2	<0.4	<0.01	<0.05

‖ Rheumatic heart disease.
¶ Significant change with exercise, $p < 0.01$.
** Significant change with exercise, $p < 0.05$.
‡‡ Right ventricular pressure.

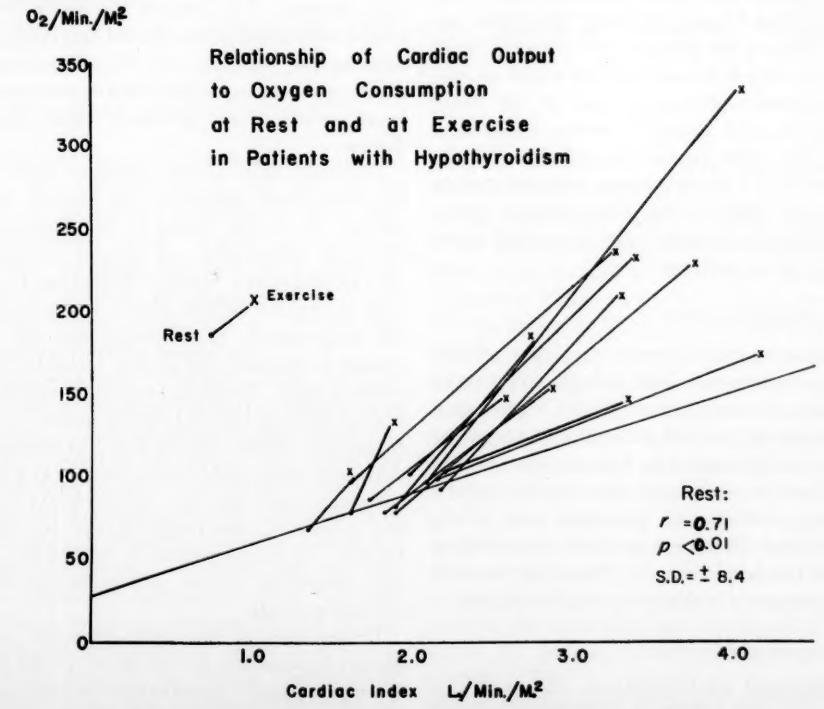

FIG. 1. RELATIONSHIP OF CARDIAC OUTPUT TO OXYGEN CONSUMPTION AT REST AND AT EXERCISE IN PATIENTS WITH HYPOTHYROIDISM

were present (E. C., 22/2/8; R. C., 30/5/13; J. P., 30/–2/6). Ventricular pressure tracings were obtained in only three of the five other patients with enlarged cardiac silhouettes but without evident effusions; in two (A. R. and E. L.) a moderate "diastolic dip" was present (31/5/10 and 28/4/8); in the third, E. D. with rheumatic heart disease, the contour was similar (38/1/8). These tracings suggest the possibility that pericardial effusion with restriction of diastolic filling may have been present in these three patients with nondiagnostic enlargement of the cardiac silhouette as well as in the three patients with presumptive radiographic evidence of effusion.

Pulmonary artery pressure

In the nine patients in whom measurements were obtained, the mean pulmonary arterial pressures were normal at rest; the mean pressure was under 10 mm. Hg in three patients with normal size of the cardiac silhouette and 15 mm. to 23 mm. in six patients with enlarged cardiac silhouettes. During exercise the mean pulmonary pressure rose to less than 15 mm. Hg in the first three patients; in three of the patients with enlarged heart shadows (A. R., I. C., and E. D.) slight pulmonary hypertension developed, and in the other three patients with enlarged cardiac silhouettes, including two with pericardial effusion (E. C., R. C., and E. L.) the pulmonary pressure rose to upper normal limits. The means for the group did not differ significantly from the normal either at rest or during exercise.

Systemic arterial pressure

The mean brachial arterial pressures at rest in these patients were normal to high with definite hypertension present in one (E. C.). The mean for the group did not differ significantly from the normal. During exercise the systolic pressure rose in all patients who increased their cardiac outputs significantly and diastolic pressures rose in six, fell in two, and did not change in the two without significant increase in output. The slight increase in mean pressure for the group was significant.

Total pulmonary resistance

The calculated total pulmonary resistance was normal in the patients with normal heart size and increased in the patients with enlarged heart contours. Again the group mean did not differ significantly from normal. There was either no change or a decrease in pulmonary resistance during exercise in all patients.

Total systemic resistance

For the group, a significant increase in calculated total systemic resistance over the normal was found at rest and at exercise. A significant decrease in resistance occurred with exercise.

DISCUSSION

Our primary interest in studying patients with hypothyroidism was to assess the circulatory dynamics in a disorder in which some of the symptoms and signs have been ascribed to congestive heart failure and in which the cardiac output is known to be at levels commonly associated with congestive failure. Observations concerning some of the physiologic changes accompanying hypometabolism have been presented and compared with normal. The present discussion will concern a comparison of the clinical and hemodynamic data in the patients with hypothyroidism with those observed in patients with typical congestive heart failure from ultimately fatal myocardial disease.

Clinically each of our patients with marked hypothyroidism presented the picture of myxedema. In several instances moderate pitting of the lower extremities was present in addition to the typical brawny edema. In contrast to patients with congestive failure, however, the edema was not progressively more severe, was not notably related to posture and always included the periorbital region. Nocturia associated with amelioration or disappearance of edema so frequent in the edematous cardiac was not noted in these patients. Although exertional breathlessness was a frequent complaint, this was not relentlessly progressive and commonly was overshadowed by exertional fatigue. In sharp contrast to most of the patients with congestive failure, orthopnea did not occur in these patients.

Enlargement of the cardiac silhouette was present on examination in two-thirds of the patients. Roentgenographic examination disclosed presumptive evidence of pericardial effusion in three of

TABLE II

*Comparison of hemodynamic data in patients with hypothyroidism with those of patients in congestive heart failure**

	Hypothyroid—12 patients		Heart disease with failure—7 patients		p	
	Rest	Exercise	Rest	Exercise	Rest	Exercise
Oxygen consumption (*ml./min./M.²*)	87 ± 3	188 ± 18†	138 ± 9	202 ± 17†	<0.01	>0.5
Cardiac output index (*L./min./M.²*)	1.88 ± 0.07	3.05 ± 0.22†	1.93 ± 0.12	2.11 ± 0.20	>0.5	<0.02
Cardiac output (*L./min.* × *100*) / Oxygen consumption (*ml./min.*)	2.18 ± 0.06	1.68 ± 0.12†	1.42 ± 0.08	1.04 ± 0.05†	<0.01	<0.05
Δ Cardiac index (*ml.*) / Δ (100 ml.) oxygen consumption	+1,285 ± 204		+304 ± 214		<0.01	
Stroke volume index (*ml./beat/M.²*)	30 ± 0.5	39 ± 0.5†	19 ± 1	17 ± 1	<0.01	<0.01
Heart rate (*beats/minute*)	64 ± 3	79 ± 5†	103 ± 5	122 ± 4†	<0.01	<0.01
A-V oxygen difference (*ml./100 ml.*)	4.66 ± 0.15	6.15 ± 0.34†	7.18 ± 0.42	9.83 ± 0.51†	<0.01	<0.01
Right atrial pressure (*mm. Hg*)	6 ± 1	7 ± 1	10 ± 3	21 ± 3†	<0.2	<0.01
Mean pulmonary artery pressure (*mm. Hg*)	15 ± 2	20 ± 2†				
Mean brachial artery pressure (*mm. Hg*)	97 ± 4	103 ± 5†	100 ± 7	110 ± 8	>0.5	<0.5
Total pulmonary resistance (*dynes cm. sec.⁻⁵*)	412 ± 62	395 ± 80				
Total systemic resistance (*dynes cm. sec.⁻⁵*)	2,577 ± 183	1,733 ± 160†	2,345 ± 204	2,518 ± 368	<0.5	<0.05

* Data are presented as means and standard errors.
† Significant change during exercise ($p < 0.01$).

these eight patients and right ventricular pressure contours, although obtained in only three of the other five, suggested the possibility of some limitation of diastolic filling. These data are consonant with the suggestion that the cardiac enlargement so common in hypothyroidism is frequently due to pericardial effusion (34–39) and is not a sign of cardiac dilatation.

The arm to tongue circulation times in these patients were in the upper normal to slightly prolonged range (15 to 25 seconds) and blood volumes were normal to low in the five patients in whom this determination was made. These observations are consonant with the numerous data contained in other reports of patients with hypothyroidism (19, 40–42) and differ from the considerably prolonged circulation times and increased blood volumes commonly found in patients with chronic congestive heart failure (43, 44). On the basis of the clinical evaluation of these patients it was felt, therefore, that in no instance could a definitive diagnosis of congestive heart failure be made.

The physiologic data obtained in these patients with myxedema differ in significant detail from those obtained in the patients with chronic congestive failure on the basis of myocardial disease as shown in Table II. At rest their cardiac outputs, although reduced to the level commonly found in the patients with "low output" congestive failure, were linearly related to the decreased oxygen consumption. The ratio of cardiac ouput to oxygen consumption did not differ from the healthy subjects. In the patients with myocardial disease and congestive failure the oxygen consumptions at rest and the basal metabolic rates were normal to somewhat elevated and hence the cardiac output was significantly lower with respect to the level of oxygen consumption than in either the patients with myxedema or healthy persons. This is reflected in the significantly higher arteriovenous difference and ratio of car-

diac output to oxygen consumption. Their stroke volumes were markedly reduced and heart rates were elevated in contrast to the patients with myxedema.

The response of the cardiac output to exercise observed in these patients with hypothyroidism was first of all qualitatively the same as that observed in healthy persons. Furthermore, although the increase in oxygen consumption during exercise was greater than usually noted in healthy persons under similar conditions, the ratio of increase in cardiac output to increase in oxygen consumption (Table I) was considerably greater than the minimum of approximately 650 ml. generally considered to be the normal response (33). Although the arteriovenous differences observed during exercise were higher than usually observed in healthy persons, the differences were not significant nor were the ratios of cardiac output to oxygen consumption during exercise different from those observed in the normal subjects. By contrast, in the patients with myocardial disease and congestive failure, the cardiac output either did not increase significantly or actually decreased during exercise. Any increase was due to tachycardia since stroke volume index remained unchanged. Since oxygen consumption showed the expected increase the arteriovenous differences became even more abnormal. The arteriovenous difference during exercise was greater in every patient in failure than the maximum value of 8.38 volumes per cent (A. R.) noted in the patients with hypothyroidism.

The reduced cardiac output in the patients with hypothyroidism was associated with normal to slightly elevated pressures in the right atrium, pulmonary artery, and brachial artery and with considerably increased calculated total systemic resistance. During exercise, despite a significant increase in both brachial and pulmonary arterial pressure, a marked drop in calculated systemic resistance and no change in pulmonary resistance occurred, as is usually observed in normal persons. In the patients with congestive failure, on the other hand, the elevated atrial pressures became significantly more abnormal despite the failure of cardiac output to increase, and calculated systemic resistance did not decrease. Pulmonary arterial pressures and calculated total pulmonary resistances in patients with congestive failure are high at rest and become more abnormal during exercise (45–47). It is suggested that in hypometabolism the somewhat higher than expected right atrial, right ventricular end diastolic and pulmonary arterial mean pressures, and the presence of diastolic dips on the right ventricular pressure tracings found in patients with enlarged cardiac silhouettes may well reflect the effects of pericardial effusion and not the presence of myocardial failure.

Patient E. D., a 41 year old woman, is of particular importance. She had rheumatic heart disease with the physical findings of mitral insufficiency and stenosis. She had been in typical congestive heart failure three years prior to study and required digitalis and diuretic therapy. One and a half years prior to study she had discontinued medication without return of symptoms. At about this time myxedema became evident clinically. On examination, despite the evidence of rheumatic heart disease with left and right ventricular and left atrial enlargement confirmed at fluoroscopy, she presented no evidence of congestive failure. Her physiologic studies at rest did not differ from the other patients. During exercise, however, her cardiac output rose only 462 ml. per 100 ml. increase in oxygen consumption, a value lower than in any other patient. This finding, which is suggestive of a decreased myocardial reserve, together with the history and other clinical data, suggests that her myocardial reserve was perhaps adequate for the reduced metabolic load of hypothyroidism but had not been adequate for that of her previous euthyroid state.

The hemodynamic data obtained in these twelve patients with hypothyroidism corroborate the clinical decision that congestive heart failure was not present and strongly suggest that, with the possible exception of the patient with overt rheumatic heart disease, no significant myocardial impairment was present.

A review of other clinical (1–4, 6, 10) and physiologic (22) studies made in patients reputed to have congestive failure attributed to myxedema has not revealed data which differ importantly from those obtained in our 12 patients. Furthermore, other data have been obtained in patients with myxedema which suggest that the circulation is competent. Although glomerular filtration rate and renal plasma flow are reduced as they are

in patients with chronic congestive failure, Davies, MacKinnon, and Platts (21) found that while a diet containing 10 Gm. of salt per day caused a rapid increase in weight and venous pressure in patients with congestive failure, a similar salt load was handled normally by patients with myxedema. In the light of these data it would appear that the physiologic protection afforded by the decreased circulatory demands of the hypothyroid state in our patients overshadowed in significance any morphologic changes in the myocardium associated with their myxedema.

SUMMARY

1. Clinical and hemodynamic studies made in 12 patients with myxedema have been compared with those obtained in 7 euthyroid patients with chronic congestive failure from primary myocardial disease.

2. In the hypothyroid patients the low cardiac outputs increased and the elevated systemic resistances decreased normally with exercise in contrast to the patients with congestive failure.

3. The mean arteriovenous oxygen difference in the patients with myxedema was not significantly different from that found in the healthy subjects at rest or during exercise. In the patients with congestive heart failure, abnormally widened arteriovenous differences became yet more abnormal during exercise.

4. In view of the normal responses of cardiac output and arteriovenous differences to exercise in the patients with myxedema, the finding of slightly elevated mean pressures in the right atria and pulmonary arteries, end diastolic right ventricular pressures, and diastolic dips in the right ventricular pressure curves in the patients with myxedema with enlarged cardiac silhouettes suggested the possibility of pericardial effusion rather than cardiac dilatation as a cause of the enlarged cardiac contour.

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BINDING OF CORTICOSTEROIDS BY PLASMA PROTEINS. III. THE BINDING OF CORTICOSTEROID AND RELATED HORMONES BY HUMAN PLASMA AND PLASMA PROTEIN FRACTIONS AS MEASURED BY EQUILIBRIUM DIALYSIS [1, 2]

By WILLIAM H. DAUGHADAY with the technical assistance of IDA KOZAK

(From the Metabolism Division, Department of Medicine, Washington University School of Medicine, St. Louis, Mo.)

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The physical state of the corticosteroid hormones in human plasma has been under investigation in this laboratory. Dialysis equilibrium experiments have been reported which show that the 17-hydroxycorticosteroids of plasma are bound to plasma proteins to an extent of more than 90 per cent (2). Of the plasma protein fractions tested for their cortisol binding power with high concentrations of cortisol, albumin had the greatest binding affinity. The importance of albumin was apparently further supported by equilibrium paper electrophoresis conducted with cortisol-4-C^{14} in which the greatest concentration of radioactivity was associated with the albumin component of plasma (3). It was difficult, however, to explain the greater binding of 17-hydroxycorticosteroids in plasma than that which occurred in equilibrium dialysis experiments using cortisol and albumin. This discrepancy has prompted a thorough reinvestigation of corticosteroid binding at physiologic levels of hormone by plasma and plasma protein fractions. Progress has been greatly aided by the availability of labeled steroids, greatly simplifying the analytical techniques. Evidence indicating the presence of two independent corticosteroid binding systems in plasma is presented in this paper.

METHODS

Heparinized blood was obtained from normal adult laboratory workers and students in midmorning following a light breakfast. In most experiments plasma was used for binding studies on the day that it was drawn, although little loss of binding affinity occurred after storage at −10° C. The conditions of dialysis were the same as previously reported (2). Usually 10 ml. of plasma in a cellophane bag (Visking nojax, 18/32 casing) was dialyzed against 40 ml. of Krebs phosphosaline buffer, pH 7.4 (4), in a large test tube. Where a high degree of binding was anticipated, 80 ml. of buffer was used. In several experiments where the amount of plasma or plasma protein fraction was limited, 5 ml. of protein solution was dialyzed against 40 ml. of buffer. The radioactive and nonradioactive steroids were included in the dialysis buffer by diluting an alcoholic solution of the steroids with buffer. The final alcohol concentration of alcohol was below 1 per cent in all experiments. Although equilibrium conditions are reached for all practical purposes in 24 hours, the dialyses were allowed to continue for 48 hours at 4° C.

The buffer was separated from the plasma at the conclusion of the dialysis and the volume measured. It was then extracted three times with ethyl acetate using 1.0, 1.0, and 0.5 volumes of solvent. The extracts were pooled and evaporated to dryness in a boiling flask. The protein concentration of the plasma was measured by a biuret method (5). The plasma was extracted a single time with five volumes of chloroform in a stoppered tube. Emulsions were broken by centrifugation and the supernatant plasma was removed by suction. Anhydrous sodium sulfate was used to dry the extract and an aliquot, usually 20 ml., was evaporated to dryness in a small boiling flask.

The steroid residues were carefully dissolved in 1.5 ml. of ethyl alcohol and then 0.5 ml. of 8 per cent glucose was added and mixed well. Duplicate aliquots of this solution were pipetted into tared brass cups containing disks of lens paper. The cups were reweighed after drying under an infrared light. Radioactivity was determined with a windowless gas flow counter operating in the proportional range. The observed counts were corrected to infinite thinness using an experimentally determined curve prepared with cortisol-4-C^{14}. The glucose provides a smooth adhesive glaze. Recovery of added cortisol-4-C^{14}, using these extraction and counting techniques, has varied between 90 and 105 per cent. Control experiments in the absence of plasma have failed to show significant adsorption of the labeled steroids on the glass or dialyzing membranes.

[1] Reported in part at the Annual Meeting of the Central Society for Clinical Research, November, 1956 (1).

[2] This investigation was supported by a research grant, C-255, from the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health, Public Health Service.

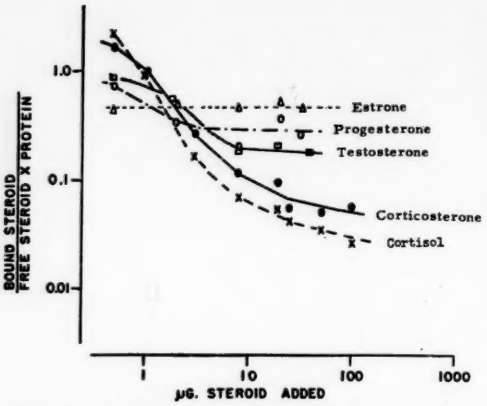

FIG. 1. CHANGES IN THE BINDING AFFINITY OF NORMAL HUMAN PLASMA FOR CORTISOL-4-C^{14}, CORTICOSTERONE-4-C^{14}, PROGESTERONE-4-C^{14}, TESTOSTERONE-4-C^{14}, AND ESTRONE-16-C^{14} IN THE PRESENCE OF INCREASING AMOUNTS OF TOTAL STEROID IN A STANDARD DIALYSIS EQUILIBRIUM SYSTEM CONTAINING 10 ML. OF PLASMA AND 80 ML. OF BUFFER

Corticosterone-4-C^{14} and cortisol-4-C^{14}, with specific activities of 1.467 millicuries per millimole, were obtained from the Endocrinology Study Section of the National Institutes of Health. Progesterone-4-C^{14}, with a specific activity of 1.63 millicuries per millimole, was prepared by Tracerlab, Boston, Massachusetts. Estrone-16-C^{14} and estradiol-16-C^{14}, with specific activities of 0.74 millicuries per millimole, were supplied by Charles E. Frost Co., Montreal. Testosterone-4-C^{14}, with a specific activity of 1.44 millicuries per millimole, was obtained from the Ray Chem Laboratories, Elmsford, New York.

The nonradioactive steroids were either in crystalline form or as a fine crystalline suspension in talc (U. S. P. Reference Standard Steroids). The steroids were extracted from the latter preparations with ethyl alcohol and diluted to the desired concentration with the dialysis buffer.

The combining affinity of a protein solution for a given steroid under the specified conditions of protein and steroid concentration has been expressed in the following way:

$$C \text{ (combining affinity)} = \frac{\text{Bound steroid}}{\text{Unbound steroid} \times \text{protein}}$$

Unbound steroid: This is the steroid which is not bound to protein as determined by the steroid concentration of the buffer corrected for the lower water content within the dialysis bag.

Bound steroid: The steroid bound to protein is the difference between the concentration of total steroid within the dialysis bag and the concentration of free steroid.

Protein: The concentration of protein within the bag after dialysis is expressed as milligrams per milliliter.

This expression has the form of an association constant but cannot be calculated in molecular terms because the molecular weights of the binding proteins other than albumin are unknown. For most of the steroids studied C is not constant for human plasma but is greatly influenced by the concentrations of the steroids in the system. This simple formulation provides an empiric method for comparing the binding observed with plasma and plasma protein fractions of different protein and steroid concentrations.

RESULTS

The binding of progesterone-4-C^{14}, corticosterone-4-C^{14}, cortisol-4-C^{14}, testosterone-4-C^{14}, and estrone-16-C^{14} by human plasma has been measured in the presence of different amounts of carrier steroid added to the dialysis equilibrium system. The changes in combining affinities with increasing concentrations of steroid have been plotted on a double logarithmic scale in Figure 1. At the lowest concentrations studied, the two corticosteroid hormones (cortisol and corticosterone) are bound nearly completely to the plasma proteins. When more steroid is added to the system there is a large decrease in the affinity of binding. After a transition phase, further additions of cortisol and corticosterone make relatively little difference in the measured affinity. The behavior of estrone in comparable experiments is quite different in that the binding affinity is not significantly influenced by the amount of estrone added to the system. The results with progesterone

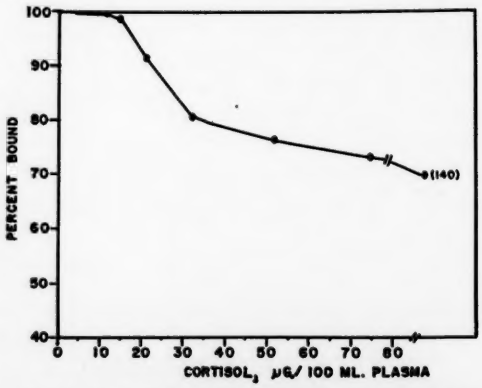

FIG. 2. THE CALCULATED CHANGES IN THE PERCENTAGE OF CORTISOL BINDING BY HUMAN PLASMA PLOTTED AGAINST THE TOTAL CORTISOL CONCENTRATION OF PLASMA

These calculations were made from the data summarized in Figure 1.

TABLE I

*A comparison of the ability of related steroids to inhibit the binding of cortisol-4-C¹⁴ **

Competing steroid	Amount	Plasma	F-4-C¹⁴†		Protein	C‡
			Free	Bound		
	μg.		cpm/ml.	cpm/ml.	mg./ml.	
None	0	A	4.1	297	66.1	1.095
		B	3.3	262	60.7	1.33
		D	3.7	245	61.5	1.08
9α-fluorocortisol	10	B	5.9	254	63.7	0.68
	10.2	D	4.7	252	62.8	0.86
d, l-aldosterone	10	C	5.8	225	60.3	0.64
3α, 11β, 17α, 21-tetrahydroxy-pregnane-20-one	10	A	7.2	248	67.1	0.51
11β, 17α, 21-trihydroxy-pregnane-3, 20-dione	10	A	9.3	237	68.8	0.37
Progesterone	8.7	D	12.4	208	61.3	0.27
Cortisone	10	B	15.9	205	59.9	0.22
11α-hydroxycortisol	10	B	15.2	222	61.2	0.24
11-desoxycorticosterone	10	A	25	179	71.1	0.10
17α-hydroxyprogesterone	9.14	C	25.5	138.5	61.7	0.080
Prednisolone	9.97	C	29.5	125.7	60.8	0.070
11-desoxycortisol (Compound S)	10	B	36.9	121.1	60.6	0.054
21-desoxycortisol	9.57	C	29.1	97.8	61.4	0.055
Corticosterone	10	A	32	113	66	0.053
2-methylcortisol	10.4	D	32	106	64	0.053
Cortisol	10	A	34.4	110.6	69	0.048
	10	B	38.5	106.5	63	0.044
	10	C	32.3	92.3	64	0.045
	10	D	31.5	98.0	61.4	0.051

*Conditions—Ten ml. of plasma dialyzed 48 hours against 40 ml. of Krebs phosphosaline buffer with 0.5 μg. of cortisol-4-C¹⁴ plus indicated amounts of competing steroid. The table is a compilation of studies on four separate plasmas which are identified for direct comparisons.

† F-4-C¹⁴ = Cortisol-4-C¹⁴.

‡ C = Combining affinity.

and testosterone are intermediate between those obtained with estrone and the corticosteroid hormones. Minor difference between the curves cannot be considered significant because the experiments were conducted with different samples of normal human plasma.

The data on cortisol binding have been recalculated and presented in a form which is more easily interpreted physiologically (Figure 2). In the graph, the percentage of the total plasma cortisol bound to plasma proteins has been plotted against the total plasma cortisol concentration. Certain assumptions were made for these calculations. The initial concentration of cortisol of this plasma was assumed to be identical with the measured concentration of 17-hydroxycorticosteroids (7.5 μg. per 100 ml.). The degree to which water entered the dialysis bag was assumed to be 5 per cent of the original volume.[3] These data permitted a calculation of the specific activity in the system. From this calculation the concentration of cortisol in the plasma at the end of the dialysis was found.

The binding curve for cortisol shows that virtually all the cortisol is protein bound when the total cortisol concentration is below 15 μg. per 100 ml. of plasma. The percentage of bound steroid decreases rapidly between 15 and 30 μg. per 100 ml. of plasma, and more slowly thereafter.

The abrupt decrease in affinity of plasma for cortisol and corticosterone with addition of carrier steroid, as illustrated in Figure 1, has permitted a study of the specificity of the binding site for cortisol. The ability of a number of structurally related steroids to inhibit the binding of cortisol-4-C¹⁴ has been examined. In the experiments summarized in Table I the degree of binding of 0.5 μg. of cortisol-4-C¹⁴ by plasma has been determined alone or in the presence of the indicated amounts of the steroids listed. The results are tabulated in an array with the steroids which block the binding of cortisol-4-C¹⁴ to the

[3] Recently, the entrance of buffer into 10 ml. of plasma has been found to average 12 per cent of the original volume. Recalculation of the data with this figure leads to no significant alteration in Figure 2.

TABLE II

*A comparison of the ability of related steroids to inhibit the binding of corticosterone-4-C¹⁴**

Competing steroid	Amount	Plasma	B-4-C¹⁴† Free	B-4-C¹⁴† Bound	Protein	C‡
	μg.		*cpm/ml.*	*cpm/ml.*	*mg./ml.*	
None		A	1.0	311	64	4.86
		C	1.8	249		2.11
d, l-aldosterone	10	B	2.8	221	60	1.33
Androsterone	8.02	C	1.8	249	65	1.14
11-dehydrocorticosterone (Compound A)	9.5	A	5.4	297	65	0.85
Progesterone	8.7	B	7.8	226	60	0.48
11-desoxycorticosterone	9.14	A	17	244	66	0.21
11-desoxycortisol (Compound S)	9.5	A	24	197	65	0.12
Cortisol	10	A	28	171	65	0.095
Corticosterone	9.57	A	30	165	63	0.086
		B	21	132	60	0.104

*Conditions—Ten ml. of plasma dialyzed 48 hours against 40 ml. of Krebs phosphosaline buffer containing 0.5 μg. of corticosterone-4-C¹⁴ plus indicated amounts of competing steroid. The table is a compilation of measurements on three separate plasmas which are identified for direct comparison.

† B-4-C¹⁴ = Corticosterone-4-C¹⁴.

‡ C = Combining affinity.

greatest extent at the bottom and those which block the binding of cortisol the least at the top.

Table I provides considerable information concerning the specificity of the binding site of plasma which has the highest affinity for cortisol. The increased ability of 11-desoxycorticosterone and 17-hydroxycorticosterone to displace cortisol-4-C¹⁴, as compared to progesterone, indicates that the hydroxyl group at either the 17 or 21 carbon positions increases the ability of a corticosteroid to displace cortisol. The possession of a second hydroxyl group at position 11, 17, or 21 by a corticosteroid possessing one such group further increases the affinity of the compound for the cortisol binding site. For example, less cortisol-4-C¹⁴ is bound in the presence of corticosterone than with desoxycorticosterone; less cortisol-4-C¹⁴ binding occurs in the presence of compound S as compared to 17-hydroxyprogesterone. It is noteworthy that a steroid possessing hydroxyl groups at all three positions (postions 11, 17, and 21), namely cortisol, only decreases the binding of cortisol-4-C¹⁴ slightly more than a corticosteroid with only two such hydroxyl groups. In these experiments the magnitudes of the effects induced by the three positions of hydroxylation were similar.

Further evidence for the comparative specificity

TABLE III

*Binding of cortisol-4-C¹⁴ by plasma protein fractions**

Protein	Source	Protein	Free F	Bound F	C†
		mg./ml.	*cpm/ml.*	*cpm/ml.*	
$II_{1,2,3}$	Protein Fndn.	32	54	8	0.005
III	Protein Fndn.	(44)‡	54	19	0.008
IV-1	Protein Fndn.	22	49	60	0.055
IV-4§	Protein Fndn.	27	15	708	1.7
V	Protein Fndn.	27	31	84	0.10
IV (No. 347)‖	Cutter Labs.	40	6	206	0.87
IV-1 (No. 17437)‖	Cutter Labs.	28	6	226	1.26
Normal human plasma		(mean of 7)			2.08

*Conditions—Four per cent protein solutions were prepared from lyophilized preparations in Krebs phosphosaline buffer. Denatured proteins were removed by centrifugation. Ten ml. of solution was dialyzed against 40 ml. of Krebs phosphosaline buffer containing 0.5 μg. of cortisol-4-C¹⁴.

† C = Combining affinity.

‡ The protein determination of this fraction is questionable because of the turbidity of the solution which developed with the Lowry method (13).

§ Only 3 ml. of protein solution was available for dialysis. This was dialyzed against 40 ml. of Krebs phosphosaline buffer.

‖ Eighty ml. of Krebs phosphosaline buffer was used for these experiments.

of cortisol binding is the unexpected finding that the configuration of the 11-hydroxyl group exerts a major influence. Substitution of the 11α-hydroxyl for the natural 11β-hydroxyl of cortisol led to a marked decrease in the ability of the unnatural compound to displace cortisol-4-C^{14} from binding by plasma. The 11-keto group likewise decreased competition.

Certain other structural changes decrease the affinity of a steroid for the cortisol binding site when evaluated in this way. These changes include the introduction of the 18-aldehydic group (compare cortisol binding in the presence of aldosterone as compared to corticosterone), the 9α-fluoro group, and the reduction of the Δ^4-3-ketone. Some structural modifications caused little change in the ability of the steroid to displace cortisol. The unsaturation at the 1 to 2 position of prednisolone and the introduction of the 2-methyl group in 2-methyl-cortisol decreased the competing power only slightly.

A more limited study of the specificity of the binding site for corticosterone-4-C^{14} has been conducted using the same method as employed in the experiments presented in Table I. The ability of related steroids to depress corticosterone-4-C^{14} binding appears to follow the same general principles described for cortisol (Table II).

The corticosteroid binding properties of lyophilized plasma protein fractions have been reexamined under the conditions used for whole plasma (Table III). The high degree of binding present in normal human plasma cannot be attributed to albumin (Fraction V). Of the fractions examined, the highest binding affinity for cortisol has been found in Fraction IV-4. A considerable difference was found in the binding exhibited by this fraction when prepared by different laboratories. Some activity was found in samples of Fraction IV and IV-1. Samples of Fraction VI have not yet been available for study.

Recently a preparation of Fraction IV-4 (Protein Foundation run No. 1033) has been studied which has a higher corticosteroid binding power than the sample described in Table III. The binding affinity of this preparation for a number of active steroid hormones has been compared with a sample of Fraction V. The contrast between the binding specificities of these two protein fractions has been presented graphically

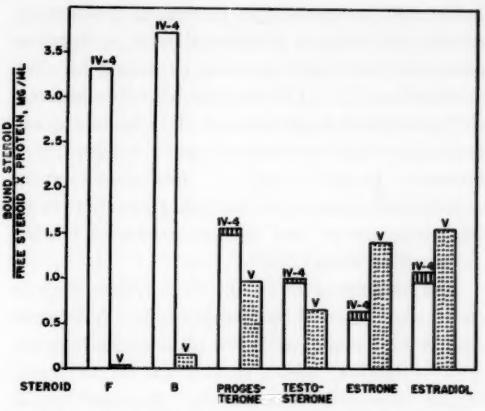

FIG. 3. A COMPARISON OF THE BINDING OF CORTISOL-4-C^{14} (F), CORTICOSTERONE-4-C^{14} (B), PROGESTERONE-4-C^{14}, TESTOSTERONE-4-C^{14}, ESTRONE-16-C^{14}, AND ESTRADIOL-16-C^{14} BY HUMAN PLASMA PROTEIN FRACTIONS V AND IV-4

The dialysis equilibrium system consisted of 5 ml. of solution containing 3.2 ± 0.2 per cent protein in buffer and 40 ml. of buffer containing 0.25 μg. of the indicated labeled steroid.

(Figure 3). Fraction IV-4 has the greatest affinity for the corticosteroid hormones (cortisol and corticosterone), and much less affinity for testosterone, estrone, and estradiol. The binding of progesterone is intermediate between these two extremes. As the sample of Fraction IV-4 probably contains as much as 15 per cent albumin,[4] a rough calculation can be made of the degree to which this contamination contributes to the observed affinity by using the formula for combining affinity previously described. The correction has been indicated at the top of the columns pertaining to Fraction IV-4. In the case of the two estrogens this is a significant fraction of the total binding affinity but this fraction is unmeasurable with cortisol and corticosterone. In contrast to Fraction IV-4, Fraction V has the greatest affinity for the estrogenic hormones and the least affinity for cortisol and corticosterone.

DISCUSSION

In an earlier publication from this laboratory, it was concluded that the degree of binding of 17-

[4] This information was provided by Dr. R. Pennell of the Blood Characterization and Preservation Laboratory of the Protein Foundation, Boston, Mass.

hydroxycorticosteroids in plasma of patients receiving cortisone or adrenocorticotropic hormone exceeded that which could be explained by albumin binding (2). The analytic difficulties inherent in the chemical determination of 17-hydroxycorticosteroids have been circumvented by the use of hormones labeled with C^{14}. The results of the experiments reported in this paper clearly indicate that there are at least two corticosteroid binding systems in human plasma.

The binding agent in the first system may be called corticosteroid binding globulin. With small amounts of cortisol added to plasma, the high degree of binding which occurs can be attributed almost entirely to this protein. It would appear significant that there is considerable specificity of the binding site for the physiologically active corticosteroids produced by the adrenal cortex. Alterations in the steroid molecule, often of a minor nature, usually decreased the ability of a steroid to inhibit the binding of cortisol-4-C^{14}. There did not seem to be any relation between the biological activity of synthetically prepared steroids and their affinity for the cortisol-4-C^{14} binding site. The amount of the corticosteroid binding globulin in plasma must be very small because the binding capacity seems to be saturated at steroid concentrations between 15 and 30 μg. per 100 ml. of plasma.

Albumin is a second corticosteroid binding protein of human plasma. The ability of albumin to bind a great number of steroids including cortisol has been abundantly established (2, 6–8). The factors determining the affinity with which albumin binds steroids differ from those which were observed with human plasma in the present experiments. Eik-Nes, Schellman, Lumry, and Samuels (6) have shown that the strength of binding by albumin was for most steroids inversely related to the number of polar groups and the degree of water solubility. Westphal (7) has observed a spectral shift in the ultraviolet absorption associated with the Δ^4-3-ketone after binding of the steroid by albumin indicating that this group probably participates in the binding. The presence of the 11-hydroxyl group weakened this interaction markedly. Hydroxyl groups at the 17 and 21 positions were without much influence (8). It is difficult to exceed the binding capacity of albumin because of the large amounts present in plasma.

The corticosteroid binding which occurred with different plasma protein fractions provides additional evidence favoring the dual nature of corticosteroid binding. The binding affinity of Fraction IV-4 for cortisol and corticosterone was much greater than that of albumin. Considerable loss of activity must have occurred during fractionation because the affinity of the most active fractions was hardly greater than that of fresh whole plasma. It would be premature to associate the corticosteroid binding activity with any particular subfraction until a higher recovery of total plasma activity is achieved. An electrophoretic separation of corticosteroid binding globulin and albumin will be described separately (9).

Recently, Sandberg and Slaunwhite (10) have published a comprehensive study of steroid binding using both ultrafiltration and dialysis equilibrium methods. They also find the greatest binding of cortisol with Fractions IV-4 and V. The results of these workers differ, however, in not indicating the predominant binding of corticosteroids by Fraction IV-4 which we have observed (Figure 3). Indeed, the binding of most of the steroid hormones examined by Sandberg and Slaunwhite was almost as great as cortisol with the exception of cortisone, which was bound significantly less. This latter finding agrees well with the limited ability of cortisone to displace cortisol from the high affinity binding site of plasma, shown in Table I. Minor differences in the experimental conditions can explain the more evident predominance of corticosteroid binding in the present paper. We have used approximately one-fifth of the amount of steroid per milligram of Fraction IV-4 employed by Sandberg and Slaunwhite. This small difference in steroid concentration can be expected to reduce the degree of binding of cortisol relative to the other steroid hormones.

With the present incomplete fractionation of plasma proteins it is impossible to state that the corticosteroid binding globulin, as defined in this paper, is a single protein distinct from other steroid binding globulins in plasma. It may be that one protein has a binding site of high affinity for corticosteroid hormones at one locus and binds a number of other hormones at other loci. The

reversible type of binding which occurs in dialysis equilibrium experiments differs from the firm binding of estrogens to protein when an incubation is carried out with liver slices in the presence of plasma as reported by Szego and Roberts (11). The significance of this latter type of binding has recently been challenged (10).

The physiologic significance of plasma proteins which bind hormones is far from clear. A similarity between the corticosteroid binding globulin and the thyroxine binding globulin (12) is suggested by these studies despite the much tighter binding of thyroxine. Binding an active hormone to a protein hardly seems an efficient way to "transport" the hormone across the capillary membrane to the cells on which hormonal action is exerted. Robbins and Rall (12) have suggested that the physiologically significant thyroxine of the plasma may be the minute concentration of unbound thyroxine rather than the proportionally much greater concentration of protein bound thyroxine. The same may be said about cortisol in that the effect exerted on the tissues may be proportional to the concentration of freely diffusible hormone. The determination of plasma 17-hydroxycorticosteroids would be, therefore, only an indirect index of the concentration of the physiologically significant unbound steroid.

SUMMARY

1. The binding of cortisol-4-C^{14}, corticosterone-4-C^{14}, progesterone-4-C^{14}, testosterone-4-C^{14}, and estrone-16-C^{14} by human plasma has been compared in dialysis equilibrium experiments. Whereas the binding of estrone is influenced relatively little by the amount of steroid in the dialysis system, the affinity of plasma for cortisol and corticosterone is greatly affected by the amount of steroid added. With additions of less than 1 μg. per 10 ml. of plasma, about 99 per cent of cortisol in plasma is protein bound. The affinity of plasma decreases abruptly with further additions of steroid. The behavior of testosterone and progesterone was intermediate between that of the corticosteroid and estrogenic hormones.

2. The ability of related corticosteroids to displace cortisol-4-C^{14} and corticosterone-4-C^{14} from the binding sites of high affinity in plasma has been examined. The presence of at least two hydroxyl groups at the 11β, 17α, or 21 positions, and an intact Δ^4-3-ketone in ring A are required for maximum displacement of the radioactive hormone. Competition is greatly decreased by the presence of the 11-keto, the 11α-hydroxyl, the 18-aldehydic or the 9α-fluoro groups on a corticosteroid molecule.

3. Dialysis equilibrium experiments with plasma protein fractions using low concentrations of cortisol-4-C^{14} demonstrated that Fraction IV-4 had the highest affinity for cortisol and corticosterone. The ability of albumin to bind these hormones under these conditions was markedly less. Fraction IV-4 had less affinity for progesterone, testosterone, estrone and estradiol than for the two corticosteroid hormones.

4. It is suggested that the high binding affinity of plasma for corticosteroid hormones in low concentrations is due to the presence of a small amount of a binding protein of high binding affinity. The binding capacity of this protein is exceeded when the concentration of cortisol rises substantially above the normal concentration. The binding of cortisol by plasma at high concentrations of hormone can be attributed largely to albumin.

ACKNOWLEDGMENTS

Plasma protein fractions used in these experiments were supplied by Dr. R. Pennell, Protein Foundation, Boston, Mass.; Dr. J. H. Hink, Cutter Laboratories, Berkeley, Calif.; and Dr. T. D. Gerlough, E. R. Squibb and Sons, New Brunswick, N. J. The steroids were generously donated by the following organizations: Ciba; Merck Sharp and Dohme Laboratories; Schering Research Division; and the Upjohn Company.

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ANNOUNCEMENTS OF MEETINGS

The Fiftieth Annual Meeting of THE AMERICAN SOCIETY FOR CLINICAL INVESTIGATION will be held in Atlantic City, N. J., on Monday, May 5, 1958, at 9:00 A.M. at the Casino Theater on the Steel Pier.

The Fifteenth Annual Meeting of THE AMERICAN FEDERATION FOR CLINICAL RESEARCH will be held in Atlantic City, N. J., at the Casino Theater on the Steel Pier on Sunday, May 4, 1958, at 9:00 A.M.

THE ASSOCIATION OF AMERICAN PHYSICIANS will hold their Seventy-First Annual Meeting in Atlantic City, N. J., at the Casino Theater on the Steel Pier on Tuesday, May 6, 1958, at 9:30 A.M. and in the Vernon Room, Chalfonte-Haddon Hall on Wednesday, May 7, 1958, at 9:30 A.M.

BINDING OF CORTICOSTEROIDS BY PLASMA PROTEINS. IV. THE ELECTROPHORETIC DEMONSTRATION OF CORTICOSTEROID BINDING GLOBULIN [1, 2]

By WILLIAM H. DAUGHADAY with the technical assistance of IDA KOZAK

(From the Metabolism Division, Department of Medicine, Washington University School of Medicine, St. Louis, Mo.)

(Submitted for publication September 30, 1957; accepted December 5, 1957)

The usefulness of equilibrium paper electrophoresis to detect the binding of corticosteroids by plasma proteins was described in an earlier paper of this series (2). Plasma was dialyzed initially against the electrophoretic buffer to which carbon labeled corticosteroids had been added. Later this buffer was used to saturate the electrophoretic paper. Thus, the concentration relationship between the buffer steroid and the protein bound steroid was maintained throughout the development of the electrophoresis. Consequently, there was little tendency for the bound steroid to trail the advancing protein components as occurs with customary electrophoresis of steroids (3). In the experiments carried out with this method using barbital buffer, pH 8.8, both corticosterone-4-C^{14} and cortisol-4-C^{14} appeared to migrate with the albumin peak of plasma.

Subsequent experiments with carbon labeled steroids using dialysis equilibrium suggested the possibility that there were two important corticosteroid binding proteins of human plasma (4). The first of these binding systems probably accounts for nearly all the binding of cortisol at normal physiologic concentrations. Under such circumstances about 99 per cent of the cortisol in plasma is protein-bound. As the concentration of hormone in the plasma rises, the degree of binding falls rapidly at first and then very much more slowly. Like plasma, plasma Fraction IV-4 has also been demonstrated to have a high affinity for corticosterone and cortisol. The affinity of this fraction for progesterone, testosterone, estrone, and estradiol is much less than for cortisol and corticosterone when tested under conditions of low hormone concentration.

The second corticosteroid binding system is operative at high plasma concentrations of cortisol. Most of this binding can be attributed to albumin whose binding characteristics have been adequately described (5–7). Cortisol and corticosterone are bound much less firmly by albumin than the other steroid hormones mentioned above.

The failure to observe two corticosteroid binding zones in our equilibrium paper electrophoresis experiments can be attributed to the fact that these experiments were carried out with amounts of corticosterone and cortisol far in excess of those needed to saturate the corticosteroid binding globulin. Binding, predominantly by albumin, could be anticipated under these conditions. It was not possible to carry out equilibrium paper electrophoresis at physiologic cortisol concentrations because the small amount of plasma which can be applied to the electrophoretic paper limits the amount of radioactive steroid available for counting. In the present paper, this difficulty was circumvented by using continuous flow paper electrophoresis of equilibrated plasma, which has permitted the demonstration of a corticosteroid binding globulin that is distinct from human albumin.

METHODS

Serum was collected from laboratory workers and medical students, two to three hours after breakfast. Before subjecting this serum to continuous flow electrophoresis, it was dialyzed extensively against the electrophoretic buffer in the presence of the radioactive steroid under study. Ten ml. of serum in a Visking cellophane bag was dialyzed against 1,500 ml. of buffer. The amount of steroid added to the serum and buffer was estimated to provide about 0.5 μg. of steroid bound to the serum and an equilibrium concentration in the buffer. To speed the attainment of equilibrium, 0.5 μg. of the labeled steroid was added to the plasma directly. To the buffer was added 2.3 μg. of cortisol-4-C^{14} or corticosterone-4-C^{14}.

[1] A preliminary report of these findings has been published (1).

[2] This investigation was supported by a research grant, C-255, from the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health, Public Health Service.

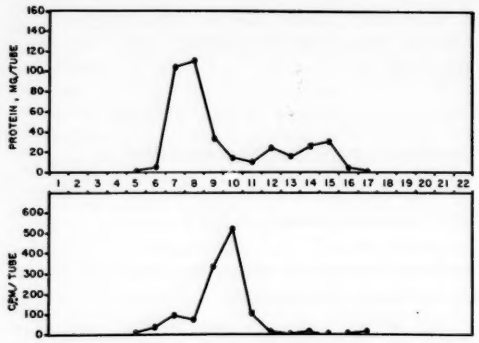

FIG. 1. CONTINUOUS FLOW ELECTROPHORESIS OF HUMAN SERUM CARRIED OUT WITH BARBITAL BUFFER, pH 8.8, IONIC STRENGTH 0.045 IN THE PRESENCE OF CORTICOSTERONE-4-C^{14}

Serum was applied to curtain above tube 14. The elution tubes are indicated by the Nos. 1 through 22 between the two curves.

In the experiments with progesterone-4-C^{14}, 1.87 μg. of this steroid was added to the buffer. Nonradioactive cortisol, 350 μg., was added in certain experiments with progesterone-4-C^{14}.

Continuous flow electrophoresis was carried out using the Karler-Misco apparatus at 4° C. Two electrophoretic buffers were used—barbituric acid-sodium barbiturate, pH 8.8, ionic strength 0.045; acetic acid-sodium acetate, pH 5.2, 0.02 M. A fractionation plate, similar to that described by Shetlar, Cahill, Stidworthy, and Shetlar (8), gave improved separation of the protein fraction. The curtains were made from Schleicher and Schuell paper No. 588. The upper reservoirs of the apparatus, which provide the flow of buffer for the curtain, were filled with the buffer which had been dialyzed against the serum in the presence of the radioactive steroid. The electrode vessels were filled with stock buffer. As soon as the curtain was wet with buffer, the current was turned on for three to five hours to create a steady state. In the barbital buffer the current flow was 13 milliamperes with 700 volts and 4 to 6 milliamperes with 800 to 900 volts with the acetate buffer.

Serum was applied to the paper through a 2 mm. wick cut from Schleicher and Schuell paper No. 470A. In a period of from 36 to 40 hours, 5 to 10 ml. of serum were fractionated. The eluent was collected in 22 small test tubes. Each tube contained between 3 and 4 ml. of eluent at the end of a run. The curtain was removed and dried rapidly with a hair drier. Drying was completed and the proteins fixed to the paper by heating to 100° C. for 30 minutes in an oven. The positions of the protein bands were identified by staining with bromphenol blue.

The eluent tubes were made up to a total volume of 5 ml. each. Aliquots were analyzed for protein using the method of Lowry, Rosebrough, Farr, and Randall (9). The protein bound hexose was determined in several experiments by the method of Winzler (10). Radioactivity was determined in each tube by extracting 3 ml. of eluent with 25 ml. of chloroform in a large stoppered tube. The extracts were washed with 2.5 ml. of 0.1 N sodium hydroxide and 2.5 ml. of water. Twenty ml. of the final chloroform extracts was evaporated in 40 ml. centrifuge tubes. The residues, of negligible mass, were transferred to small brass cups in alcohol. Radioactivity was determined in a gas flow counter operating in the proportional range.

The cortisol-4-C^{14} and the corticosterone-4-C^{14} used in these experiments were obtained from the Endocrinology Study Section of the National Institutes of Health. Both compounds had a specific activity of 1.467 millicuries per millimole. The progesterone-4-C^{14} was obtained from Tracer Lab, Inc., and had a specific activity of 1.63 millicuries per millimole.

The general reproducibility of the electrophoretic experiments was excellent, although slight changes in the rate of migration and the exactness of definition occurred. The accuracy of counting was better than ± 10 per cent.

RESULTS

The binding of cortisol-4-C^{14} was first examined electrophoretically in barbital buffer. When the electrophoresis was performed in a buffer with an ionic strength of 0.045, the peak of radioactivity of the bound steroid followed the main albumin peak (Figure 1). Only a slight asymmetry with a shoulder of increased radioactivity in the albumin containing tubes was apparent. The position

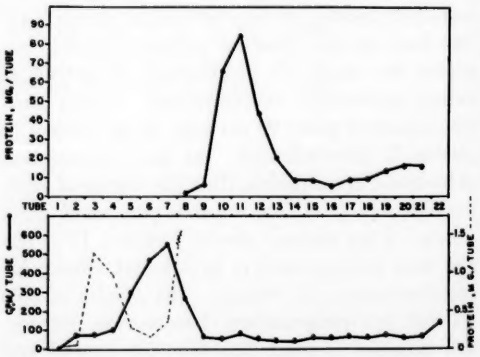

FIG. 2. CONTINUOUS FLOW ELECTROPHORESIS OF HUMAN SERUM IN THE PRESENCE OF CORTICOSTERONE-4-C^{14}

Acetate buffer, pH 5.2, 0.02 M was used. Serum was applied to curtain above tube 12. The dotted line on the lower graph indicates protein concentration measured by the Lowry, Rosebrough, Farr, and Randall procedure (9).

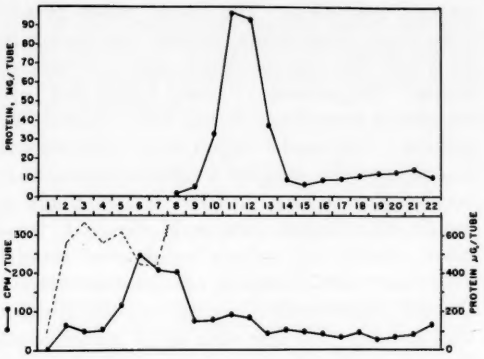

FIG. 3. CONTINUOUS FLOW ELECTROPHORESIS OF HUMAN SERUM IN THE PRESENCE OF CORTISOL-4-C^{14}

Acetate buffer, pH 5.2, 0.02 M was used. Serum was applied to curtain above tube 12. The dotted line on the lower graph indicates the protein concentration measured by the Lowry, Rosebrough, Farr, and Randall procedure (9).

of the radioactivity in respect to the albumin peak was influenced by minor experimental variations. Lowering the ionic strength of the barbital buffer increased the mobility of the bound radioactive steroid. With a buffer of ionic strength of 0.02, the bound steroid slightly preceded the albumin peak.

To gain further information concerning the binding of corticosterone, experiments have been conducted using 0.02 M acetate buffer, pH 5.2. Albumin migrates little at this pH and descends directly from the point of application above tube 12. In control experiments in which cortisol-4-C^{14} was added directly to the curtain in the absence of serum the steroid descended somewhat cathodally from the point of application with peak concentrations in tubes 14 and 15. The displacement of the noncharged steroid can be attributed to electroendosmotic movement of the buffer. When the experiment was carried out with serum the distribution of radioactivity was very different from the control experiment (Figure 2). The greatest concentrations of the labeled cortisol were located in tubes 5, 6, and 7. These tubes are on the anodal side of the albumin which was concentrated in tubes 10 and 11. The tubes containing the greatest amounts of the labeled steroid were found to contain only small quantities of protein.

When the electrophoresis of normal serum was carried out with cortisol-4-C^{14}, a similar distribution of radioactivity was observed (Figure 3). In this particular experiment there was a greater concentration of the anodally migrating protein components in the region of the cortisol binding than observed in the experiment with corticosterone.

The binding pattern with progesterone-4-C^{14} is of considerable interest (Figure 4) in that there is a clearly defined double peak of binding of radioactivity. One peak is associated with the anodally migrating component which binds corticosteroids, but in addition there is significant binding in the region of the albumin peak. This finding is consistent with the interpretation of the binding of progesterone-4-C^{14} in dialysis equilibrium experiments with different quantities of carrier nonradioactive progesterone (4). It was suggested that the binding of progesterone was relatively less for corticosteroid binding globulin and relatively greater for albumin as compared to cortisol. To support this view, the ability of cortisol to displace progesterone-4-C^{14} from the corticosteroid binding globulin has been studied. In the electrophoretic experiments, depicted in Figure 5, 350 μg. of nonradioactive cortisol was added to the preliminary dialysis carried out with 10 ml. of serum, 1,500 ml. of acetate buffer and

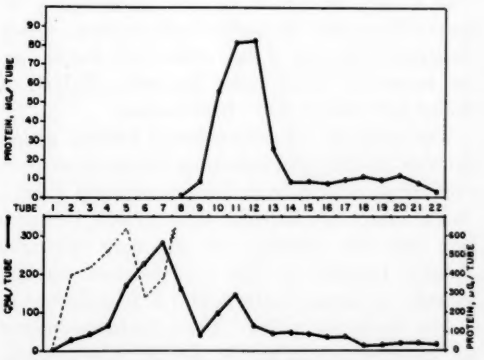

FIG. 4. CONTINUOUS FLOW ELECTROPHORESIS OF HUMAN SERUM IN THE PRESENCE OF PROGESTERONE-4-C^{14}

Acetate buffer, pH 5.2, 0.02 M was used. Serum was applied to curtain above tube 12. The dotted line on the lower graph indicates protein concentration measured by the Lowry, Rosebrough, Farr, and Randall procedure (9).

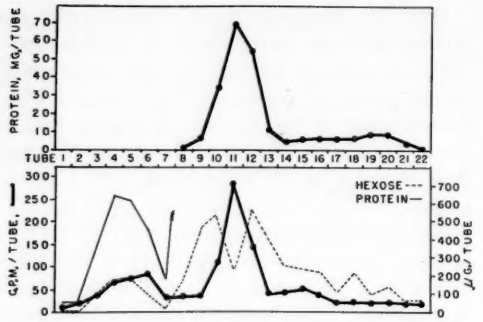

FIG. 5. CONTINUOUS FLOW ELECTROPHORESIS OF HUMAN SERUM IN THE PRESENCE OF PROGESTERONE-4-C^{14} AND NONRADIOACTIVE CORTISOL.

Acetate buffer, pH 5.2, 0.02 M. was used. Serum applied above tube 12.

progesterone-4-C^{14}. Under these conditions, the binding of the progesterone-4-C^{14} by the anodally moving protein was virtually eliminated and the binding by the albumin peak greatly accentuated. Analyses of the bound hexose distribution are also included in this graph.

DISCUSSION

The application of the principle of equilibrium electrophoresis to continuous flow electrophoresis has presented no particular problems. The apparatus selected for use has been favorable for such studies because of the small capacity of the reservoirs feeding the buffer to the curtain. Other designs of this type of instrument have larger buffer reservoirs which make the initial dialysis of buffer with serum more troublesome.

The existence of corticosteroid binding globulin with mobility characteristics distinct from albumin seems evident from the experimental results. In the customary barbital buffer system this protein has the mobility of an alpha globulin. Anodal mobility of the corticosteroid binding globulin in acetate buffer, pH 5.2, is indicative of a low isoelectric point. These features suggest an acidic glycoprotein. At this pH, two anodally migrating glycoproteins have been recognized. The fastest component has been described by Mehl, Golden, and Winzler (11) as M-1 and the second component with a mobility only slightly faster than albumin has been called M-2. The thyroxine binding globulin is believed to be associated with M-2. The fastest minor protein peak in our experiments probably corresponds to M-1, and M-2 was not clearly separated from albumin. The presence of bound hexose and hexosamine in these fractions has been confirmed by analysis. The anodal migration of corticosteroid binding globulin occupies a position intermediate between that reported for M-1 and M-2, and in certain experiments, such as in Figure 1, these tubes contain only minute amounts of protein. This observation cannot be construed as evidence that the corticosteroid binding material is other than protein. The ease with which corticosteroid binding globulin of plasma is saturated with small additions of corticosteroids indicates that only minute amounts of the protein are normally present. The preliminary binding experiments which have been done with plasma protein fractions indicate that corticosteroid binding globulin may be contained in Fraction IV-4, a fraction known to be rich in glycoproteins (2).

The relatively low affinity of progesterone for the corticosteroid binding globulin has been experimentally useful in permitting the demonstration of binding of this protein and albumin. The change in distribution of progesterone with the addition of cortisol is explained by the higher affinity of the corticosteroid binding globulin for cortisol.

SUMMARY

1. The binding of cortisol-4-C^{14}, corticosterone-4-C^{14}, and progesterone-4-C^{14} by human serum proteins has been studied by continuous flow electrophoresis. Careful predialysis of the serum, the steroid, and the electrophoretic buffer has prevented elution artifacts.

2. With barbital buffer, pH 8.8, the peak of corticosterone binding migrates more slowly than albumin and is present in the alpha globulin region. In acetate buffer, pH 5.2, the corticosteroid binding globulin moves anodally away from the albumin component.

3. Binding studies conducted with progesterone-4-C^{14} have permitted the demonstration of binding both by the anodally moving binding protein and by albumin. The bound progesterone could be displaced off the anodally moving component onto the albumin peak by adding cortisol to the system.

4. These observations provide direct evidence of the existence of a corticosteroid binding globulin which is separate from albumin and suggest its glycoprotein nature.

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MECHANISM OF THE GLUCOSURIA PRODUCED BY THE ADMINISTRATION OF STEROIDS WITH GLUCOCORTICOID ACTIVITY [1]

By E. RUDOLPH FROESCH,[2] ALBERT I. WINEGRAD,[3] ALBERT E. RENOLD, AND GEORGE W. THORN

(From the Departments of Medicine, Harvard Medical School and the Peter Bent Brigham Hospital, Boston, Mass.)

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Routine methods for the measurement of glucose in urine are based on the reducing properties of this sugar or on its effects upon the plane of polarized light. With these methods the daily excretion of "glucose" in urine averages approximately 1 Gm. in normal subjects. It has always been appreciated that in normal subjects only a small percentage of the total substances so measured actually represented glucose. The identity of glucose, however, has been established by procedures based on fermentation, glucosazone formation, or chromatography (1, 2); these procedures are poorly suited for quantitative analysis at low glucose concentrations. Recently, a specific and simple enzymatic method for the measurement of glucose in urine has become available and it has been established that young, healthy subjects consistently excrete a small quantity of glucose averaging 115 mg. per day and rarely exceeding 200 mg. per day (3). This rate of urinary glucose excretion is relatively independent of variations in the dietary carbohydrate intake. The acute administration of carbohydrate-active steroids, however, leads almost invariably to a significant increase of glucose excretion (3–6). In most normal subjects this augmented glucosuria does not exceed 3 Gm. In patients with diabetes it may reach 100 to 150 Gm. It is the purpose of this study to investigate the mechanism by which this increased glucosuria is produced.

Glucose titration studies have been carried out in normal subjects on control days and during the administration of prednisone in doses known to produce significant glucosuria. The glucose titration procedure was selected since, *when repeated in the same individual and under standard conditions,* this procedure permits the detection of changes in glucose tolerance and of the renal handling of gradually increasing glucose loads, in addition to measuring glomerular filtration rate and maximal tubular reabsortive capacity for glucose. Renal glucose clearance has also been studied in two patients with Cushing's syndrome with evident disturbance in carbohydrate metabolism. Finally, the effect of cortisol on renal glucose clearance has been studied in two patients with renal glucosuria. These individuals, both of whom exhibited significant glucosuria at fasting blood glucose levels, offered the opportunity of studying the effects of steroids with carbohydrate activity under fasting conditions.

MATERIAL AND METHODS

All studies were carried out on the Metabolic Ward of the Peter Bent Brigham Hospital. Four healthy young male volunteers served as normal subjects. The glucose titrations were started between 9 and 10 a.m. after a fast of 12 hours. Adequate hydration was achieved by the administration of water by mouth. The subjects remained in a semirecumbent position throughout the study. After the urine flow had reached 8 or more ml. per minute, an inulin prime was injected intravenously followed by a sustaining infusion of inulin in 0.6 per cent salt solution at the rate of 8.5 ml. per minute. In addition, 200 ml. of water was given by mouth every 30 minutes to maintain adequate hydration and urine flow. Constancy of the rate of infusion was insured by the use of a Bowman infusion pump. The rate of infusion was regularly checked on the graduate cylinder containing the infusion solution. After an equilibration period of 45 or more minutes the glucose titration was begun by infusing three successive solutions containing identical amounts of inulin but increasing concentrations of glucose (to result in glucose administration rates

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[2] Recipient of a Fellowship from the American Diabetes Association.

[3] Postdoctoral Fellow of the United States Public Health Service.

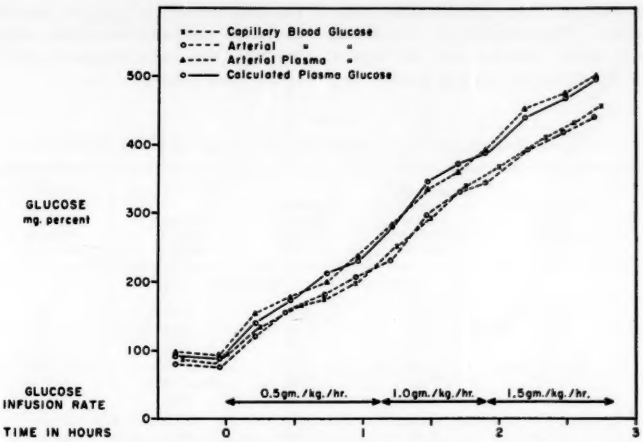

FIG. 1. VALIDATION OF THE CORRECTION FACTOR USED TO OBTAIN ARTERIAL PLASMA GLUCOSE LEVELS FROM CAPILLARY BLOOD GLUCOSE LEVELS

Measured (capillary glucose, arterial blood glucose, arterial plasma glucose) and calculated (calculated plasma glucose) values obtained during a glucose titration study in a normal subject.

of 0.5, 1.0, and 1.5 Gm. per Kg. per hour). These solutions did not contain electrolytes other than the small amount of sodium chloride which is present in inulin preparations.

Urine was collected at intervals of 15 minutes through an indwelling catheter. The bladder was not rinsed since the urine flow exceeded 8 ml. per minute (7). Duplicate capillary blood samples for glucose determina-

TABLE I

Glucose titration studies carried out in three normal male subjects before and during the administration of prednisone

	Control I	Control II	Prednisone I	Prednisone II
Subject W. E. (age, 33 years; weight, 82 Kg.; height, 179 cm.)				
Total glucose infused (Gm.)	212	218	213	210
Total glucose excreted (Gm.)	21.0	23.0	44.6	39.7
Glomerular filtration (ml./min.)*	123 ± 2	131 ± 1	138 ± 1	134 ± 3
Maximal glucose reabsorption (ml./min.)*	247 ± 13	277 ± 17	248 ± 5	280 ± 8
Blood glucose level at which significant glucosuria first occurred (mg. %)	167	178	170	184
Subject J. P. F. (age, 36 years; weight, 64 Kg.; height, 178 cm.)				
Total glucose infused (Gm.)	177	157	177	169
Total glucose excreted (Gm.)	28.8	18.5	35.0	26.4
Glomerular filtration (ml./min.)*	137 ± 2	130 ± 1	150 ± 3	138 ± 1
Maximal glucose reabsorption (mg./min.)*	338 ± 9	306 ± 10	355 ± 4	315 ± 11
Blood glucose level at which significant glucosuria first occurred (mg. %)	197	185	183	207
Subject G. S. (age, 27 years; weight, 86 Kg.; height, 178 cm.)				
Total glucose infused (Gm.)	163		158	165
Total glucose excreted (Gm.)	18.4		26.9	23.9
Glomerular filtration (ml./min.)*	108 ± 1		127 ± 2	123 ± 2
Maximal glucose reabsorption (mg./min.)*	268 ± 18		277 ± 10	297 ± 10
Blood glucose level at which significant glucosuria first occurred (mg. %)	174–270		198	173–266

* Mean values plus or minus standard error of the mean. Number of periods averaged 12 or more for glomerular filtration rate, 6 to 8 for maximal glucose reabsorption.

tions were carefully collected from the fingertips after thorough arterialization. The sampling of urine and blood was meticulously timed. Furthermore, the studies in each subject were timed in an identical fashion, *i.e.*, the same amount of glucose was infused over the same period of time and the urine collections were made at the same time with relation to the infusion in all studies for each subject.

TABLE II

Glucose titration studies in Subject W. E. (normal male, age 31 years)

Time *min.*	Capillary blood glucose *mg./ml.*	Arterial plasma glucose (calculated) *mg./ml.*	Glomerular filtration (inulin) *ml./min.*	Glucose filtered *mg./min.*	Glucose excreted *mg./min.*	Glucose reabsorbed *mg./min.*
			Control Study I (6/25/56)			
0–15	0.86	0.96	121	116	0.1	116
15–30	0.83	0.93	130	121	0.2	121
30–45	1.02	1.14	119	136	0.5	136
45–60	1.39	1.56	122	190	0.6	189
60–75	1.67	1.87	120	224	7.4	217
75–90	1.99	2.23	116	259	25	234
90–105	2.16	2.42	129	312	44	268
105–120	2.53	2.83	139	393	109	284
120–135	2.94	3.29	126	415	154	261
135–150	3.17	3.55	118	419	162	257
150–165	3.45	3.86	119	459	213	246
165–180	3.86	4.32	119	514	310	204
180–195	4.02	4.50	125	562	379	183
			Control Study II (7/24/56)			
0–15	0.80	0.90	131	108	0.1	108
15–30	1.00	1.12	131	147	0	147
30–45	1.44	1.61	137	221	0.7	221
45–60	1.78	1.99	138	275	9.5	266
60–75	2.04	2.28	131	299	30	269
75–90	2.30	2.58	127	328	45	283
90–105	2.77	3.10	133	412	108	304
105–120	3.21	3.60	132	475	128	347
120–135	3.52	3.94	127	500	195	305
135–150	3.87	4.33	123	533	263	270
150–165	3.85	4.31	130	560	338	222
165–180	4.23	4.74	132	626	417	209
			Prednisone Study I (50 mg. prednisone 12 and 3 hours prior to study—6/26/56)			
0–15	0.96	1.08	142	153	0.1	153
15–30	1.10	1.23	147	181	0.6	180
30–45	1.70	1.90	147	279	16.5	263
45–60	1.95	2.18	135	294	58	236
60–75	2.20	2.46	131	322	82	240
75–90	2.38	2.67	137	366	119	247
90–105	2.70	3.02	140	423	147	276
105–120	3.15	3.53	131	462	221	241
120–135	3.54	3.96	143	566	325	241
135–150	4.10	4.59	138	633	386	247
150–165	4.67	5.23	133	696	444	252
165–180	5.18	5.80	138	800	548	252
180–195	5.53	6.19	136	842	614	228
			Prednisone Study II (fourth day of prednisone, 75 mg. daily—6/29/56)			
0–15	0.88	0.99	130	129	0.1	129
15–30	0.82	0.92	118	109	0.2	109
30–45	1.00	1.12	125	140	0.2	140
45–60	1.51	1.69	122	206	0.9	205
60–75	1.84	2.06	137	282	8.1	274
75–90	2.10	2.35	140	329	30	299
90–105	2.20	2.46	150	369	48	321
105–120	2.74	3.07	132	405	106	299
120–135	3.14	3.52	146	514	253	261
135–150	3.40	3.81	130	495	234	261
150–165	3.70	4.14	133	551	286	265
165–180	4.38	4.91	144	707	432	265
180–195	4.76	5.33	136	725	455	270

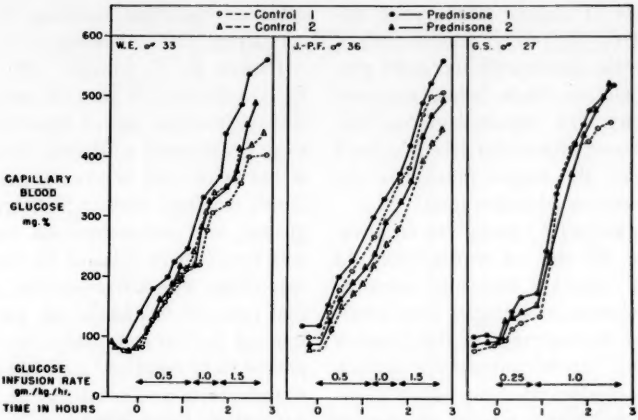

FIG. 2. CHANGES IN BLOOD GLUCOSE DURING GLUCOSE "TITRATION" IN THREE NORMAL SUBJECTS BEFORE AND DURING PREDNISONE ADMINISTRATION

Control 1 and 2: control studies. Prednisone 1: study carried out after 12 hours of prednisone administration. Prednisone 2: study carried out after four days of prednisone administration.

Blood and urine glucose determinations were carried out in duplicate according to the method of Froesch and Renold (3). Blood and urine inulin were determined in duplicate by a modification of the method of Roe after pretreatment with glucose oxidase to remove the interfering glucose from all samples (8). Highly purified glucose oxidase preparations which did not give rise to any significant blank in the Seliwanoff color reaction were kindly supplied by Dr. V. Auerbach (9) from the Department of Biological Chemistry, Harvard Medical School, and by Dr. Underkofler from the Takamine Laboratories, Clifton, New Jersey.

Calculations were made according to Smith, Goldring, Chasis, Ranges, and Bradley (10). For the calculation of filtered glucose load and of tubular glucose reabsorption, the blood glucose values were corrected for the different water content of whole blood and plasma. This was done according to Nichols and Nichols (11), assuming in all studies a hematocrit value of 45 per cent. A validation of this correction was obtained during a glucose titration study in which arterial blood samples were obtained, permitting the determination of plasma glucose as well as blood glucose (Figure 1). The mean figures given for maximal glucose reabsorption capacity are based in all studies on the final five to eight periods at which the filtered glucose load exceeded the maximal reabsorption capacity, while the mean figures for glomerular filtration rate represent all measurements made during each study.

RESULTS

Studies in normal subjects

Eleven glucose titration studies were carried out in three normal subjects, first on one (Subject G. S.) or on two control days, then following the administration of 100 mg. of prednisone in two oral doses of 50 mg. each, 12 and 3 hours before the beginning of the infusion, and finally on the fourth consecutive day of prednisone therapy at the dose level of 75 mg. daily by mouth. The results of these studies are summarized in Figure 2 and Table I. In addition, the detailed protocol of the four studies carried out in Subject W. E. is presented in Table II, to illustrate the procedure in more detail. Apparent differences in the profile of the glucose titration curves (Figure 2) should be checked against the total amount of glucose actually infused in each instance (Table I).

The following summarizing statements appear justified: (*a*) The administration of prednisone led to increased glucosuria in all instances, and this effect was greater after 12 hours than after 4 days of prednisone action. (*b*) Glomerular filtration rate was increased over the individual control values in five out of six studies carried out during prednisone administration. (*c*) Decreased glucose tolerance (as evidenced by higher blood glucose values during identical glucose loading) was observed in all subjects 12 hours after the beginning of prednisone therapy. However, glucose tolerance returned toward or to normal on the fourth day of administration of the drug. (*d*) In no instance was a significant decrease noted

in the maximal rate of glucose reabsorption capacity, as a result of prednisone administration, and in no instance did significantly increased glucosuria occur at glucose loads below maximal reabsorption capacity. (*e*) Impaired glucose tolerance and/or increased glomerular filtration rates adequately explained the excess glucosuria observed during prednisone administration.

Subject A. W. (Table III) was given an intravenous infusion of 200 mg. of cortisol over 12 hours; glomerular filtration rate and maximal tubular glucose reabsorption capacity were measured 10 hours after the beginning of this infusion and on a control day. In this subject the infusion of cortisol did not significantly alter either measurement. The high control rate of glomerular filtration should be noted.

Studies in patients with Cushing's syndrome

Maximal tubular glucose reabsorption has been measured in two patients with Cushing's syndrome presenting definite evidence of impaired carbohydrate metabolism. In these studies glucose titrations have not been carried out since both patients had grossly elevated fasting blood glucose levels. A constant amount of glucose was infused instead and the results of these studies are presented in Table III. The values obtained for maximal glucose reabsorption capacity in both patients were well within the normal range.

Studies in patients with renal glucosuria

Two studies have been carried out in patients with renal glucosuria. Both patients excreted glucose in the fasting state and thus provided an opportunity to study the effects of glucocorticoids on the renal handling of glucose without concurrent glucose loading.

Patient E. T. (height, 146 cm.; weight, 46 Kg.), a dwarfed 30 year old male, had known glucosuria from the age of nine and, in addition, tubular reabsorption defects for phosphate and amino acids, and severe osteomalacia. This patient's maximal tubular reabsorption capacity for glucose was determined on two occasions and was found to be 32 and 30 mg. per minute, respectively, with corresponding glomerular filtration rates of 54 and 52 ml. per minute. At the time of the present study, his renal disease appeared to be relatively stationary since glomerular filtration rate and renal plasma flow (PAH) had not further deteriorated over the last 10 years and since the osteomalacia seemed at least stationary and perhaps improved by clinical and roentgenographic criteria. Patient F. N., a 22 year old healthy young male (height, 163 cm.; weight, 73 Kg.), was found to excrete glucose in the urine upon routine medical examination. An intravenous glucose tolerance test was normal. The diagnosis of renal glucosuria was established by the measurement of a maximal tubular glucose reabsorptive capacity of 130 mg. per minute. Glucose excretion on 10 control days averaged 16 Gm. per day. No other renal anomaly could be detected and no evidence for any additional metabolic disturbance was found.

Both patients were kept fasting for 16 hours previous to cortisol administration and throughout the entire study. Inulin clearance was measured over 16 hours in Patient E. T. and, after the first 6 hours, 200 mg. cortisol was added to the infusion. In Patient F. N. inulin was infused over 10 hours, inulin clearance was measured over 8

TABLE III

Measurement of maximal glucose reabsorptive capacity in one normal subject (before and after cortisol) and in two patients with Cushing's syndrome

	Number of periods	Glomerular filtration inulin† (*ml./min.*)	Maximal glucose reabsorption† (*mg./min.*)
Subject A. W. (male; age, 21 years; weight, 75 Kg.; height, 175 cm.)			
Control study	5	168 ± 3	391 ± 2
After cortisol*	6	173 ± 1	377 ± 2
Patient E. R. (female; age, 34 years; weight, 61 Kg.; height, 158 cm.)	7	101 ± 2	273 ± 3
Patient M. C. (female; age, 47 years; weight, 98 Kg.; height, 156 cm.)	8	149 ± 3	289 ± 11

* Cortisol, 200 mg., administered intravenously over 12 hours; measurements started at 10 hours.
† Mean value plus or minus standard error of the mean.

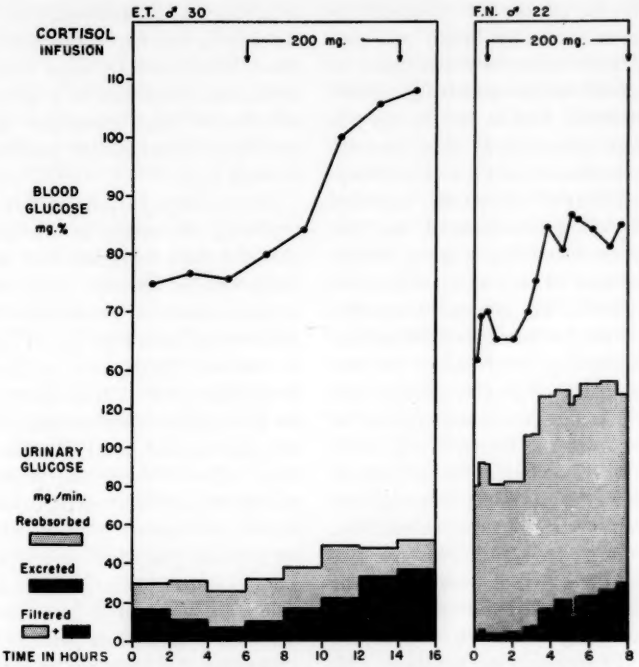

FIG. 3. THE EFFECT OF CORTISOL ON BLOOD GLUCOSE AND RENAL GLUCOSE CLEARANCE IN TWO FASTED PATIENTS WITH RENAL GLUCOSURIA

hours and, after 3 control periods of 10 minutes each, 200 mg. cortisol was added to the infusion and administered over the remaining 7 hours and 30 minutes. The results of both studies are shown in Figure 3. Over a period of eight hours, while the patients were fasted and while glucose was lost in the urine in considerable quantities, blood glucose rose from 75 to 108 mg. per cent in Patient E. T., and from 62 to 87 mg. per cent in Patient F. N. In addition, glomerular filtration rate increased in both patients and the rate of urinary glucose loss rose approximately threefold (from 10 to 35 mg. per minute) in Patient E. T. and eightfold (from 4 to 32 mg. per minute) in Patient F. N. as a result of the cortisol infusion. Tubular glucose reabsorption showed some irregular variations in both patients, but no significant or consistent increase or decrease.

DISCUSSION

The average figures for maximal glucose reabsorption capacity in four normal male subjects reported in this study are somewhat lower than those previously reported by Smith and co-workers (10). This difference may perhaps be attributed to the specific method for the determination of glucose in blood and urine used in this study. In earlier studies blood and urine glucose were measured by methods based on the reducing properties of glucose. With these procedures the non-glucose reducing substances present in blood during glucose loading account for a significant portion of the "glucose" measured, and the blood "glucose" levels obtained under these conditions may exceed the true values by as much as 30 to 50 mg. per cent. This would result in observed glucose filtration and reabsorption values slightly higher than the true ones.

It is necessary to discuss the somewhat surprising finding that glucose reabsorption showed a tendency to decline in the later periods of the titration studies despite a continued rise in the filtered glucose load and despite the lack of significant concomitant alterations of the glomerular filtration rate. This phenomenon was quite constant in each study carried out in Subjects G. S.

(Figure 4) and W. E. (Table II). Similar observations have been made by Smith and colleagues (10) and were tentatively attributed to the selective closure of certain sensitive glomeruli as a result of intrarenal edema due to the administration of large quantities of water and saline. Such an explanation would not seem to apply under the conditions of the studies reported here since the only saline administered was that required to supply the sustaining quantity of inulin in the early portions of each study before the administration of glucose was begun. Moreover, the total quantity of fluid administered did not approach that administered by Smith and co-workers (10) in the studies in which similar results were obtained. It should be recalled, however, that the transfer of glucose from the lumen of the renal tubule to venous renal blood must involve one or more enzyme systems and that a given maximal rate of glucose reabsorption reflects the maximal rate of these reactions. It is quite reasonable to believe that one or the other of these enzymatic reactions could be depressed temporarily during a glucose titration during which a steady state is never achieved. Progressive overloading with glucose as well as the many physiological responses to this loading may well produce temporarily significant alterations of the intracellular environment so closely connected with many aspects of enzyme action. To cite but one example, progressive glucose loading represents a major stimulus for increased insulin secretion; it has been stated (12), although not fully substantiated, that insulin can depress tubular glucose reabsorption. We, therefore, must bear in mind that the physiologic responses to a prolonged intravenous infusion of large quantities of glucose may in themselves influence the functions we are trying to study.

The primary purpose of this study was that of exploring the origin of the glucosuria that follows the acute administration of compounds with glucocorticoid activity. It is well known that the administration of glucocorticoids or of adrenocorticotropic hormone (ACTH) frequently leads to elevated fasting and postprandial blood glucose levels (4–6). It is also well established that the glomerular filtration rate often increases during glucocortoid administration (13, 14). Both these factors increase the filtered load of glucose and their combined effect could be sufficient to exceed the normal tubular reabsorption capacity for glucose. In addition, a depression of tubular glucose reabsorption, as a result of glucocorticoid action, has frequently been invoked. This effect has been described in patients with essential hypertension (15) and in premature infants (16) treated with ACTH and cortisone. Other investigators, however, have failed to demonstrate a depression of maximal glucose reabsorption capacity after the administration of ACTH and cortisone to normal subjects (17) and patients (18, 19).

In the studies reported here the administration

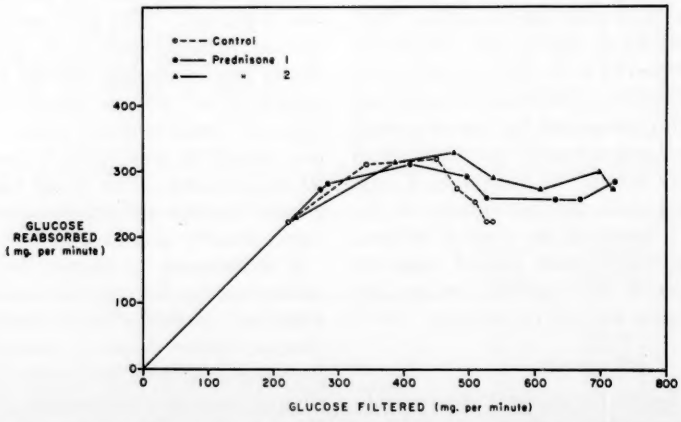

FIG. 4. CHANGES IN GLUCOSE REABSORPTION WITH INCREASING FILTERED GLUCOSE LOAD IN A NORMAL SUBJECT BEFORE AND DURING PREDNISONE ADMINISTRATION

of either prednisone or cortisol uniformly resulted in either some impairment of glucose tolerance, or an increased rate of glomerular filtration, or both. The glucocorticoid-induced increased glomerular filtration rate was more striking in the subjects with relatively low initial values. When glucose titration was performed four days as well as 12 hours after the beginning of prednisone administration, glomerular filtration rate remained elevated whereas glucose tolerance was less impaired after four days then had been the case after 12 hours of hormone action. As a result, the proportion of administered glucose excreted in the urine was least in the control studies, highest in the studies carried out 12 hours after initiation of prednisone administration, and intermediate or almost normal on the fourth day of prednisone therapy. The improved glucose tolerance on the fourth day accounted for the observed decrease in glucosuria and is in agreement with data collected during the administration of prednisone for two consecutive days to 30 healthy males. In this instance a greater quantity of glucose was, as a rule, excreted on the first day of therapy. The improvement in glucose tolerance on continued glucocorticoid therapy has been attributed to mechanisms of counter regulation among which a compensatory increase in insulin secretion is likely to play a significant part. The combined effects of prednisone or cortisol on glucose tolerance and on glomerular filtration were adequate to explain the increased glucosuria. *In no instance did the administration of prednisone or cortisol result in a lowered maximal glucose reabsorption capacity or in increased glucosuria at glucose loads below the maximal reabsorption rate.*

The studies in the two patients with a limited and relatively fixed tubular capacity to reabsorb glucose again failed to show any further lowering in tubular glucose reabsorption as a result of cortisol administration. In addition, these two studies should be discussed with regard to their contribution to the characterization of the action of cortisol on carbohydrate metabolism *in man*. Both patients were subjected to a preliminary fast of 16 hours in the face of continued loss of glucose in the urine. Under these conditions the utilization of glucose by tissues such as muscle is known to decrease to a minimum (20) and blood glucose needed for utilization by tissues such as brain is provided almost exclusively by hepatic gluconeogenesis. It is of great interest to find that under these conditions the administration of cortisol resulted, within two to four hours, in a highly significant increase in *both* blood and urine glucose. Since an initial decrease in blood glucose was not observed it is likely that the renal effect is secondary to the extrarenal effect. The extrarenal effect, on the other hand, must almost of necessity primarily consist of an increased hepatic gluconeogenesis. These observations *in man* are, of course, in agreement with the more definitive studies which have been carried out in animals (21–23), both as to effects and as to the time required for their appearance. The authors are well aware of further studies, carried out under quite different conditions, and which have been interpreted as suggesting additional glucocorticoid effects on some phase of glucose utilization (24–26).

In two patients with Cushing's syndrome and "steroid diabetes" measurements of maximal glucose reabsorption capacity were within the normal range, but more information will be needed in order to state that tubular glucose reabsorption is usually normal in Cushing's syndrome. Since glucose titrations were not carried out, no statement can be made with regard to glucose excretion at lower glucose loads in these two patients.

SUMMARY

1. The mechanism of the glucosuria produced by the administration of steroids with glucocorticoid activity has been investigated in four healthy young males using a specific enzymatic method for the determination of glucose in blood and urine. Each subject served as his own control. Glucocorticoid administration resulted in impaired glucose tolerance and an increased rate of glomerular filtration. The combination of these two factors adequately accounted for the glucosuria produced. In no instance was the maximal glucose reabsorption capacity affected significantly, nor was increased glucose excretion noted at glucose loads below the maximal reabsorption capacity.

2. Two patients with renal glucosuria leading to measurable urinary glucose excretion at fasting blood glucose levels were given intravenous infusions of cortisol over 10 hours during a prolonged fast. Blood glucose increased markedly in both subjects and urinary glucose excretion in-

creased from 10 to 35 mg. per minute and from 4 to 32 mg. per minute, respectively. The simultaneous increase in both urine and blood glucose levels during cortisol administration and while fasting is best interpreted as suggesting an increased rate of hepatic gluconeogenesis. Increased glomerular filtration contributed to the increased urinary glucose excretion. Tubular glucose reabsorption was not significantly altered.

3. Maximal glucose reabsorption capacity was measured in two patients with Cushing's syndrome and glucosuria and was within normal limits in both instances.

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FURTHER STUDIES ON THE "PHARMACOLOGY" OF PLACEBO ADMINISTRATION [1]

By LOUIS LASAGNA, VICTOR G. LATIES, AND J. LAWRENCE DOHAN

(From the Department of Medicine (Division of Clinical Pharmacology), and the Department of Pharmacology and Experimental Therapeutics, Johns Hopkins University School of Medicine, Baltimore, Md.)

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The effects produced when presumably inert substances (*e.g.,* lactose and saline) are given to normal and diseased individuals are appreciated by many. Few physicians would deny the power of a placebo to influence pain, anxiety, or other "subjective" states in certain individuals; the ability of placebos to affect "objective" phenomena (such as vomiting) or to produce "side effects" of various sorts has also been reported (1–3).

This article is an attempt to describe certain lesser known aspects of the "pharmacology" of the placebo by depicting, in some detail, the ways in which the clinical use of inert substances may lead to effects which are usually considered to be the exclusive property of active agents. Certain implications of the data will be discussed.

METHODS AND RESULTS

Time-effect curve in single dose experiments

One of the basic indices of pharmacologic activity is the time-effect relationship. When an active drug is given to patients, a maximal effect is typically achieved at a certain point in time. It is not widely appreciated that placebos can also show this behavior.

In Figure 1 are plotted some data obtained in a study of the effects of aspirin or a placebo on postpartum pain. One hundred and twenty-eight patients were studied in an obstetrical ward during the five day period following delivery. Identical appearing capsules were administered at random to any patient requesting medication for pain. All patients were interviewed by the same technician under double blind conditions immediately prior to medication, and at stated intervals after medication, and asked how bad their pain was. Four arbitrary pain categories were used; "very severe," "severe," "moderate," and "slight," plus another category for "no pain." Data were obtained at one-half, one, two, and three hours after medication. The average pain relief scores were obtained as follows: Each patient was given a pain relief score for each interview after medication. If a patient described no relief at all at a given interview, a score of zero was recorded; if a decrease in pain was reported, a score of one, two, three or four was given, depending on the degree of relief reported. Thus, a change from "very severe" to "severe," or from "severe" to "moderate," or from "slight" to "no pain" counted as "one"; a change from "very severe" to "moderate," "severe" to "slight," or "moderate" to "no pain" counted as "two," and so forth. The sum of scores for all patients at a given interview point was then divided by the number of patients to give a mean pain relief score for the one-half hour point, the hour point, and so forth. These data are plotted in Figure 1. The general similarity in the shape of the curves for those patients receiving aspirin and for those receiving placebo is evident, although the two treatments differ considerably in efficacy. A total pain relief score was obtained for each subject by adding the relief scores at each interview point. A mean total pain relief score was then calculated by averaging all such scores for each drug. The mean total pain relief score for aspirin over the first three hours after medication was 5.91 with a standard error of 0.35; for placebo, 3.45 plus or minus 0.44. This difference is significant at the 0.01 level. In analyzing the placebo curve to see whether there was indeed changing efficacy with time, a two-tailed sign-test (4) was applied to the 23 patients who showed different degrees of relief at the one-half hour and one hour points. Of these, 20 showed a greater degree of relief at the one hour point than

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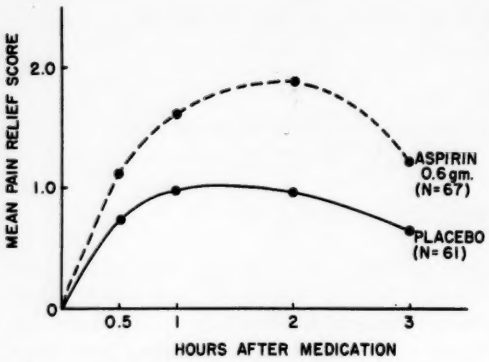

FIG. 1. MEAN PAIN RELIEF SCORES AFTER PLACEBO OR ASPIRIN IN PATIENTS SUFFERING FROM POSTPARTUM PAIN

at the half-hour interview, a difference significant at the 0.01 level. Although the mean score for placebo relief is somewhat lower at three hours than at one hour, this difference is not statistically significant.

"Cumulative" effects of placebos

A second basic type of pharmacologic study is the delineation of the effect of repeated doses of a drug. This is often considered to be a reflection of increasing concentration of drug in the blood or body. It is not generally appreciated that placebos can also show a "build-up" in effect, and that there may be a "carry-over" after cessation of placebo therapy.

Presented in Figures 2 and 3 are the data from two separate experiments. In one experiment, 34 patients suffering from tuberculosis, and on antituberculosis drugs, were given a yellow tablet daily for seven days, having been told that the tablets would increase their "appetite" and improve their "pep and energy." [2] They were asked to indicate their status each day on sheets, as shown in Table I. For four days after medication was stopped, additional observations were made. The results, shown in Figure 2, indicate that the placebo therapy was associated with gradual rises in subjective reports of both appetite and pep. The levels achieved were maintained after cessation of placebos. In order to provide a baseline against which the reliability of the changes could be tested, the nine ratings before placebos were begun were averaged. A comparison of the mean of the first three ratings made under placebo with this baseline showed a rise in appetite scores from 2.51 to 2.93, which was statistically significant at the 0.01 level [sign-test, two-tailed (4)]. For pep the change was from 2.72 to 2.91. This change was not statistically significant. For both variables the curves continued to rise, however, as the placebo therapy continued. Appetite rose from 2.93 to 3.45 and pep from 2.91 to 3.34, if the means of the first three days' ratings on placebo are compared with those of the last three of the placebo therapy. Both of these changes are significant at the 0.01 level. The curves did not drop following the end of placebo therapy, the rating four days later being 3.53 for appetite, 3.50 for pep.

Figure 3 illustrates similar data on a population of 31 hospitalized patients suffering from various chronic illnesses. Here there was a short pre-placebo period of three days, a placebo period of six days, and a post-placebo period of two weeks. (There was, however, a lapse of five days during the post-placebo period when no interview data were obtained.) Once again there appears to be a cumulative beneficial effect over the period of placebo administration, with a partial return to pre-placebo status in the case of "pep" two weeks after stopping placebos. The mean ratings for the pre-placebo period were 3.05 for

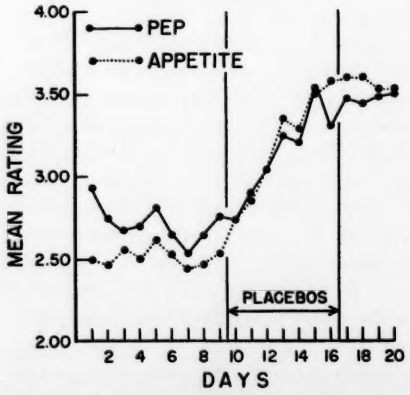

FIG. 2. PEP AND APPETITE RATINGS IN 34 TUBERCULOUS PATIENTS TREATED WITH PLACEBO

[2] It should be emphasized that the responses dealt with here are *verbal reports*. The extent, *e.g.*, to which food intake would have been correlated with "appetite" is an interesting but different question.

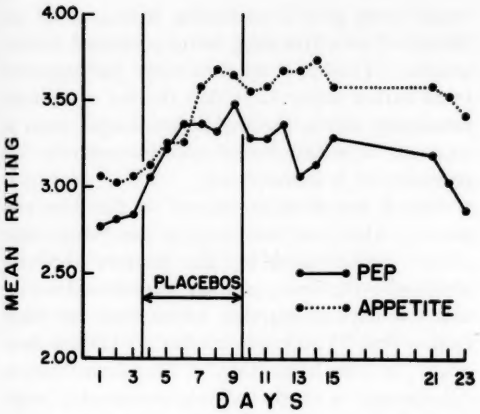

FIG. 3. PEP AND APPETITE RATINGS IN 31 PATIENTS HOSPITALIZED FOR VARIOUS CHRONIC DISEASES AND TREATED WITH PLACEBO

appetite and 2.78 for pep. Both rose to a mean of 3.22 for the first three days on placebo. Sign tests showed that only the change for pep was significant ($p < 0.01$). The ratings rose further to means of 3.63 and 3.39 for appetite and pep, respectively, for the last days of placebo therapy. The increase for appetite is statistically significant at the 0.01 level. As in the first study, the ratings remained at substantially the same levels after placebos were withdrawn. The mean ratings over the next six days were 3.64 for appetite and 3.24 for pep. Two weeks after the stopping of placebos, pep had shown a decrease to a mean of 3.03 for the last three ratings available. This drop differs significantly from the 3.39 of the last three days on placebo. The ratings for appetite did not show such a decrease, with a mean for the last three days of 3.51.

Effectiveness of placebos in relation to severity of disease

Another general characteristic of drugs is the inverse relationship of their efficacy to the severity of a given complaint. The same relationship for placebos has been apparent in some of our own data. In a study on the efficacy of morphine and injected saline on postoperative pain (2), an inverse relationship existed between the number of doses of medication required postoperatively and the efficacy of morphine or placebo (see Table II).

TABLE I

Questionnaire

A) How is your appetite today? (Check one of the following)
   1. No appetite at all
   2. Poor appetite
   3. Fair appetite
   4. Good appetite
   5. Very good appetite

B) How is your pep and energy today? (Check one of the following)
   1. No pep or energy at all
   2. Very little pep or energy
   3. Fair pep and energy
   4. Good pep and energy
   5. Very good pep and energy

If the average severity of pain of a group of patients can be gauged by the ability of a given dose of morphine to relieve their pain, it would appear that the patients requiring the largest number of doses of medication had more severe pain and were less relieved by morphine and placebo. In any case, there is an interesting parallelism between the performance of morphine and the performance of placebo.

Figure 4 also bears on this point. These data were collected in the previously described postpartum study, and are a breakdown of the data on complete relief of pain observed after placebo into those observations collected on patients describing their pain initially as "very severe" or "severe" as contrasted with those obtained on patients with pain initially described as "slight" or "moderate." The percentage of patients reporting complete relief of pain at any given time after medication is higher in the case of the "slight-moderate" group than in the "very severe-severe"

TABLE II

Pain relief with morphine or placebo in patients suffering from postoperative pain *

Group	No. of Pts.	Morphine	Placebo
I (2 doses/pt.)	12	92%	58%
II (4 doses/pt.)	21	75%	40%
III (6 doses/pt.)	15	61%	40%
IV (8 or more doses/pt.)	15	58%	15%

* The patients are stratified according to number of doses of medication required during entire postoperative period.

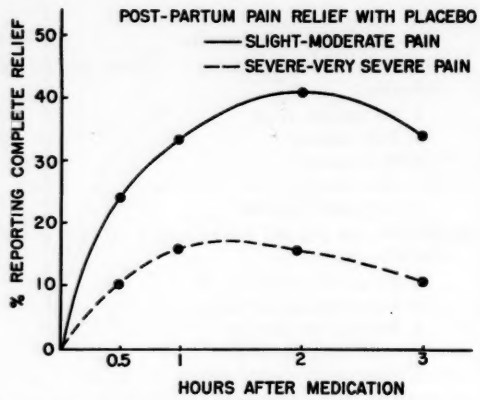

FIG. 4. RELATIONSHIP BETWEEN REPORTED SEVERITY OF PAIN AND ANALGESIC EFFICACY OF PLACEBO

group. The percentage of patients reporting complete relief of pain at some time during the three hour interval post-placebo was 21 per cent in the case of the "very severe-severe" group ($n = 19$), and 57 per cent in the case of the "slight-moderate" group ($n = 42$). This difference is significant at the 0.01 level. These data seem most easily interpreted as a less effective handling of the greater therapeutic challenge by placebo. The aspirin data from this study have been similarly analyzed and also indicate a similar lessened efficacy in patients with greater pain.

DISCUSSION

There are certain implications in these data. First of all, uncontrolled studies that claim a new or old drug to have shown unequivocal therapeutic benefit, merely because of "peak effects" or "cumulative effects" or persistent benefit after cessation of treatment, must be interpreted with considerable caution. A placebo effect is obviously not an "all or none" phenomenon, and such effects are not necessarily turned off or on, like an electric light bulb. Secondly, the time-effect relationships of placebo phenomena may be extremely important in deciding upon the times when data are to be collected in controlled trials. It is conceivable, for example, that in a certain situation the effects of suggestion are rapidly obtained, but also wear off fairly rapidly. In another situation the effects may require longer to wear off (5). A failure to collect data at points other than the placebo "peak" may give a misleading notion about the efficacy of an active drug being compared against placebo. Thirdly, it appears likely [as suggested in an earlier paper (2)] that the use of patients presenting severe therapeutic challenges may, at least on occasion, render placebo controls less necessary or less important.

Several questions are raised by the data presented. How can one explain the "cumulative effects" experienced by the patients receiving placebos? At times, patients interviewed on the first few days on placebos volunteered the information that, "You know, it takes a while for these drugs to take hold, Doc." The desire not to "disappoint" a doctor who is presumably eager for the patient to show improvement may increase progressively with the passage of time, as may the degree of reinforcement for a patient who feels that a "correct" answer (*i.e.*, improvement) will result in the doctor taking a greater interest in him.

In regard to the "carry-over" described, we can only speculate. One possibility is that patients who are improved on placebos may feel (at least temporarily) that the placebo has achieved a "cure" and that further medication is not needed. It must be difficult for a patient to believe that a doctor who had observed significant benefit in a patient from the administration of a small pill would stop or withhold such a pill if a continued need existed. One might also interpret the placebo reaction in terms of simple learning theory. Typically, in learning experiments, the abrupt withdrawal of the cue for response (here the administration of pills) is not followed by immediate cessation of response. Instead, one usually sees a gradual and irregular decline. It would be of great interest to see studies of the effectiveness of prolonged placebo therapy in various clinical situations.

It is evident that these studies give no proof that the results described were *caused* by the placebos, and would not have occurred in the absence of their administration. In regard to the pertinence of the data in recommending caution in uncontrolled experiments, such demonstration is not necessary, of course. Nevertheless, it seems unlikely that such data as presented in Figures 2 and 3, with similar curves in different populations, are really due to chance improvement unrelated

to the giving of placebos. These groups were chosen because of the relative stability of their surroundings and their illness at the time of our studies.

These data on severity of complaint and placebo efficacy are somewhat at variance with those reported by Beecher (6), who found that patients studied early in the postoperative period (where pain is presumably more severe than later) are handled almost as well by placebo as by morphine, whereas later in the postoperative course morphine performs much better than placebo. The results were interpreted as showing that placebos work most effectively when the stress is greater. However, it is of interest that Keats has found (in similar patients studied by a similar technique) placebo and morphine yielding pain relief in 27 per cent and 60 per cent, respectively, of patients early in the postoperative course, and 55 per cent and 80 per cent, respectively, late in the postoperative course (7). Keats' findings are thus in agreement with those in the present report.

SUMMARY

Data are presented to indicate that subjective responses to placebos can mimic certain characteristics of "active" drugs, such as "peak effects," "cumulative effects," "carry-over effects," and varying efficacy depending on the severity of the complaint being treated. Certain implications of these facts are discussed.

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THE PHYSIOLOGICAL MEANING OF THE MAXIMAL OXYGEN INTAKE TEST [1]

By JERE H. MITCHELL,[2] BRIAN J. SPROULE,[3] AND CARLETON B. CHAPMAN

(From the Cardiopulmonary Laboratory of the Department of Internal Medicine, University of Texas Southwestern Medical School, Dallas, Texas)

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According to Hill, maximal oxygen intake is reached when oxygen intake per unit time has attained ". . . its maximum and remains constant . . . owing to the limitation of the circulatory and respiratory systems (1)." In more definitive terms, when one subjects a normal individual to progressively increasing workloads, allowing sufficient time for recovery between each increment of work, a linear relation between workload and oxygen intake is found. Ultimately, maximal oxygen intake per unit of time is reached; beyond this point the workload can usually be increased still further but, ordinarily, oxygen intake levels off or declines.

The maximal oxygen intake which a normal individual can achieve is sometimes taken as an index to maximal cardiovascular function provided pulmonary function is normal. If this concept is sound, the test may come to be of enormous value in the critical evaluation of normal and abnormal cardiovascular function. The superiority of a test that measures cardiovascular capacity, over one that determines whether or not an individual can attain a single arbitrarily chosen workload, is apparent. A value for cardiovascular capacity should characterize a given individual precisely; use of a single workload merely places him in one of two groups. The difficulty, insofar as maximal oxygen intake is concerned, is simply that its physiological meaning is imperfectly understood. The view that cardiac capacity is the determinant of maximal oxygen intake is surmise, not established fact. Ventilatory factors, for example, are usually said not to be involved in determining maximal oxygen intake but have not really been ruled out. For that matter, the various studies that demonstrate some degree of arterial desaturation during heavy work are often thought to suggest that a pulmonary factor, either ventilatory or diffusive, may be involved. Finally, the interplay between cardiac output and AV oxygen difference during heavy work needs further scrutiny. There can be no doubt, theoretically, that the degree to which AV oxygen difference can expand is, like cardiac output, a determinant of maximal oxygen intake.

It is the purpose of this presentation to examine some of these relationships by direct measurement in the hope that the physiological meaning of the maximal oxygen intake test can be clarified.

METHOD

In order to establish normal data for this laboratory, a maximal oxygen intake test similar to that described by Taylor, Buskirk, and Henschel (2) was applied to 65 normal men. A motor-driven treadmill was used for the exercise. For normal subjects, a 10 minute warmup period (3 mph at 10 per cent grade) was followed by a 10 minute rest period. The first test run was then carried out, usually at 6 mph and zero grade. Before the treadmill was started, the subject was connected to a Rudolph two-way breathing valve by means of a rubber mouthpiece, his nose being completely closed with a nasal clamp. For the first minute of the run, expired air was used to wash out a Douglas bag connected to the expiratory side of the valve. Expired air for analysis was collected for the last minute of a two and one-half minute run. The use of this time period was based on the data of Taylor, Buskirk, and Henschel (2), Robinson (3), and Donald, Bishop, Cumming, and Wade (4). Analysis of the expired air was immediately carried out using a Beckman E-2 magnetic oxygen analyzer and a Liston-Becker infrared carbon dioxide analyzer. Duplicate analyses were carried out with a Scholander apparatus at regular intervals. The volume of expired air was measured in a Tissot gasometer and was corrected to STPD. These data permitted calculation of oxygen consumption either by using change in nitrogen content for correction of expired (to inspired) volume, or by use of a nomogram (5). Pulse rate during exercise was measured electrocardiographically. Respiratory rate was

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[2] Cardiac trainee, National Heart Institute.

[3] Research Fellow.

obtained by use of a strain gauge assembly connected to the expiratory side of the Rudolph valve. After a 10 minute rest period, the workload was increased by raising the grade to 2.5 per cent, the speed being held at 6 mph, and the procedure repeated. Subsequently, the subject performed work at increasing levels, the load being increased each time by raising the grade 2.5 per cent, until oxygen intake per minute leveled off. In most subjects, the entire test required about an hour and a half.

Ordinarily, the point at which the oxygen intake curve (plotted against workload) ceased to rise was taken as maximal oxygen intake. In most instances (72 per cent), when the workload was increased beyond that producing maximal oxygen intake, the value either remained unchanged or declined. In some cases, however, a relatively slight rise occurred, necessitating the formulation of strict criteria for deciding whether or not maximal, or near maximal, intake actually had been reached. Plots of oxygen intake against workload for the entire material (Figure 1) showed that, until a maximal value was attained, oxygen intake rose 142 ± 44 ml. with each increase in workload. If the rise was less than 142 minus 88 (twice the standard deviation), or 54 ml., the final value was accepted as the maximal intake, the assumption being that the subject had attained his true maximal value or had reached the beginning of a plateau and could not increase his intake very much more.

With most younger normal subjects, it was found to be unnecessarily time-consuming to use 2.5 per cent grade increases at the lower levels, since most of them did not attain maximal intake until grades of 10 per cent or more were reached. It was also noted that values for oxygen intake sometimes varied erratically at lower workloads, especially in anxious individuals or in those given to hyperventilation. In some young normal subjects, maximal oxygen intake was not reached even at the highest grade possible (14.75 per cent), making it necessary to increase the speed as well as the grade. The highest workload required was 9 mph at 14.75 per cent grade. With older subjects, no such problem arose. In fact, in some instances slower speeds were used if the 6 mph setting proved to be too troublesome for the subject.

In order to test the repeatability of the method as performed in our laboratory, first and second trials were done on 15 normal subjects and the data analyzed by standard statistical techniques (6).

The method for determining maximal oxygen intake was essentially unchanged when it was combined with determination of cardiac output and blood studies (15 normal subjects). Subjects for the more elaborate procedures were chosen at random. Maximal oxygen intake was first determined by the usual method. At a later date both cardiac output and maximal oxygen intake were determined at rest and at three workloads. The dye dilution technique was employed for estimating cardiac outputs. Prior to beginning the tests, a PE 90 catheter was inserted through a thin-walled 16 gauge needle into the left antecubital vein and was passed proximally to the upper brachial. Then a similar, but shorter, catheter was introduced into the brachial artery of the same arm, also through a thin-walled 16 gauge needle. Finally, the same method was used to introduce a long PE 90 catheter retrograde into the femoral vein to the midthigh level. Local anesthesia was routinely used before inserting the 16 gauge needles. The catheters were attached to three-way stopcocks and were kept filled with normal saline solution containing a small amount of heparin. For the actual measurement of cardiac output, 10 mg. of Evans blue (T-1824) was delivered at the end of the catheter in the brachial vein about one and one-half minutes after the beginning of exercise (during the collection of expired air), it having been shown by Donald, Bishop, Cumming, and Wade (4) that cardiac output and AV oxygen difference reach a steady state after the first minute of exercise. Arterial blood samples were collected at one second intervals in tubes containing powdered heparin, using either a manual collection device or an electrically operated collector. During the final 20 to 30 seconds of the exercise, blood samples were collected anaerobically from all three catheters (brachial artery, brachial and femoral veins) for measurement of oxygen tension and content, carbon dioxide content and pH. Oxygen tension was determined polarographically (7), oxygen content and carbon dioxide content by the Van Slyke and Neill method, and pH by use of a Cambridge Research model pH meter. Carbon dioxide tension was calculated from a Singer and Hastings nomogram (8).

Analysis of blood for dye content was carried out according to a previously published method (9), using a Beckman model DU spectrophotometer. The data were plotted and extrapolated by the method of Hamilton,

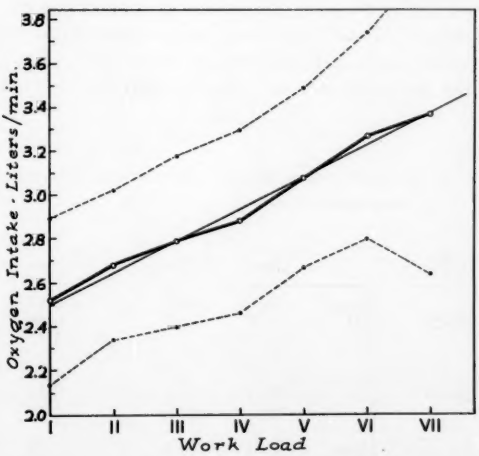

FIG. 1. RELATION BETWEEN SUCCESSIVE INCREMENTS IN WORKLOAD AND OXYGEN INTAKE IN NORMAL MEN

Heavy black line represents mean values at each load. Dashed lines represent standard deviation about each point.

TABLE I

Maximal oxygen intake in liters per minute—Duplicate trials in 15 normal men

Subject	Trial I	Trial II
A. G.	2.94	2.89
J. M.	3.86	3.77
C. D.	3.46	3.34
J. D.	2.84	2.60
J. B.	2.99	2.93
B. A.	3.34	3.39
S. W.	2.45	2.40
C. M.	3.20	3.57
C. J.	3.51	3.65
L. C.	3.46	3.23
C. McR.	2.95	3.08
W. M.	2.99	3.27
J. W.	2.77	2.52
F. H.	3.13	3.14
N. H.	2.07	2.24
Mean	3.06	3.07
S.D.	0.46	0.44

Moore, Kinsman, and Spurling (10). Mean circulation time, "central" blood volume, and cardiac output were then determined, also by the method of Hamilton and co-workers.

RESULTS

Comparisons of gas analyses done by the gas analyzers, on the one hand, and the Scholander method (11), on the other, showed the former to be highly reliable. Mean values for oxygen content in 17 gas samples chosen at random as the work proceeded were 16.38 ± 0.84 per cent for the gas analyzers and 16.42 ± 0.90 per cent for the Scholander method. Corresponding values for carbon dioxide were 4.86 ± 0.78 and 4.85 ± 0.76 per cent, respectively. The polarographic technique, as employed (7), has been shown to be highly reliable at all levels of oxygen tension. More specifically, the method yields values over physiologic venous and arterial ranges that correspond closely to pO_2 values calculated from simultaneous Van Slyke analyses and from determinations by the microbubble technique of Riley, Proemmel, and Franke (12). In very low ranges (3 to 10 mm. Hg) the error is about 5 per cent if values are calculated from the polarographic equation (7) instead of being read off a calibration curve. From 20 to 100 mm. Hg, the percentage error drops to about 2.5 per cent and is still lower at higher oxygen tensions. Repeatability of the method at relatively low oxygen tensions can be judged from first and second trials done on eight blood samples from different sources, equilibrated with known gas mixtures containing 6.1 and 12.6 per cent oxygen (42 and 89 mm. Hg partial pressure), respectively. The first trials on the mixture containing 6.1 per cent oxygen yielded a mean value of 43.2 ± 0.9 mm. Hg; the mean for the second trials was 42.9 ± 0.8 mm. Hg (n = 8 in both trials). For the second mixture, the corresponding mean values were 88.7 ± 1.3 and 89.4 ± 2.0 mm. Hg.

The repeatability of the maximal oxygen intake test, if rigid criteria for determining the point at which the maximal value has been attained are

TABLE II

Oxygen intakes in normal men of various ages at increasing workloads, including that producing maximal oxygen intake

Age group		6 mph, 0 grade		Premaximal		Maximal		Postmaximal	
		L./min.	*ml./Kg./min.*	*L./min.*	*ml./Kg./min.*	*L./min.*	*ml./Kg./min.*	*L./min.*	*ml./Kg./min*
20–29	Mean	2.59	34.6	3.15	41.8	3.37	44.7	3.18	42.7
	S.D.	0.27	2.7	0.49	4.8	0.51	3.9	0.42	4.3
	n	33		35		36		28	
30–39	Mean	2.54	32.7	2.88	37.3	3.04	39.3	2.91	36.6
	S.D.	0.31	3.2	0.38	3.3	0.39	3.3	0.54	4.6
	n	18		18		18		18	
40–49	Mean	2.51	30.4	2.79	33.6	2.94	35.4	2.77	32.9
	S.D.	0.44	2.9	0.59	4.3	0.60	3.3	0.46	3.5
	n	8		8		8		8	
Over 50	Mean			2.13	27.7	2.46	32.0	2.26	29.4
	S.D.								
	n			3		3		3	

TABLE III

Ventilation and oxygen removal rates in normal men at increasing workloads

Age group		6 mph, 0 grade		Premaximal		Maximal		Postmaximal	
		A*	B*	A	B	A	B	A	B
20–29	Mean	61.3	43.0	81.7	39.2	94.9	36.0	95.2	34.0
	S.D.	8.7	6.3	14.6	6.4	15.9	5.6	17.3	5.2
	n	33	33	35	35	36	36	28	28
30–39	Mean	66.1	39.3	80.5	36.8	89.4	35.0	88.5	33.3
	S.D.	13.3	6.4	14.9	6.8	17.3	5.7	18.8	5.7
	n	18	18	18	18	18	18	12	12
40–49	Mean	66.3	38.2	81.0	35.2	88.8	32.7	82.7	34.3
	S.D.	11.0	5.3	19.8	5.1	21.3	4.4	18.6	5.1
	n	8	8	8	8	8	8	5	5
Over 50	Mean			57.0	38.2	65.5	38.7	60.6	38.4
	S.D.								
	n			3	3	3	3	3	3

*A: Total ventilation (expired volume) in liters per min. (BTPS). B: O_2 removal rate expressed as ml. of O_2 (STPD) removed from each liter of air (BTPS) respired.

applied, is demonstrated in Table I, in which the results of first and second trials, weeks to months apart, are shown for 15 subjects. Variance analysis discloses that almost all the variance is attributable to interindividual differences; the random component is quite small and the intraindividual component virtually negligible.

The mean values for maximal oxygen intake obtained in normal men aged 20 and over are given in Table II.

In Table III are presented the mean values for ventilation and oxygen removal rates in normal men at increasing workloads including the maximal and postmaximal levels.

The values for oxygen intake, cardiac output, AV oxygen difference and other hemodynamic variables at rest and at the maximal oxygen intake load are shown in Table IV.

Table V presents mean values for arterial and venous oxygen content, oxygen tension, per cent oxygen saturation, and pH, both at rest and at maximal oxygen intake.

The values for cardiac output and AV oxygen difference at the maximal oxygen intake and at a higher workload are presented in Table VI.

DISCUSSION

The need for an effective measure of circulatory capacity is undisputed. The maximal oxygen intake test, to judge from the views of Hill (1) and of Bainbridge (13), may fill the need but its determinants have not been sufficiently clarified to enable one to accept the test as a measure of circulatory capacity with assurance. The term *maximal oxygen intake* must, itself, be viewed skeptically. It seems clear from the work of Christensen and Högberg (14) and Taylor, Buskirk, and Henschel (2) that the actual value reached for a given individual depends on the nature of the physical activity. It is, therefore, maximal relative to a given set of conditions, which must be carefully defined, rather than in an absolute sense.

For one thing, the actual technique used is far from standard. In the present work, the technique used by Taylor was followed fairly closely except that the subject was required to grip a supporting shelf with his left hand while he ran. Another difference was the time interval between each workload. In our own work, 10 to 15 minutes is allowed; Taylor, however, runs only one workload per day and requires several days to complete a full determination. Buskirk and Taylor's figure for an 18 to 29 year group of sedentary students and soldiers was 3.44 ± 0.46 liters per minute (15). For our 20 to 29 year group the corresponding figure is 3.37 ± 0.51 liters per minute. These groups were not actively in training and neither appears to have been biased toward excessively fat or excessively lean individuals. Åstrand's value for 33 Swedish athletes aged

TABLE IV

*Cardiac output, stroke volume, calculated A V O_2 differences, and other items in 15 normal subjects at rest and at maximal O_2 intake**

	Standing resting	Maximal O_2 intake level
O_2 intake (L./min.)	0.340 ± 0.04	3.22 ± 0.46
n	5	15
Cardiac output (L./min.)	5.4 ± 0.8	23.4 ± 5.5
n	14	15
Pulse rate	91 ± 17	187 ± 10
n	14	13
Stroke volume (ml.)	62 ± 18	125 + 25
n	13	13
AV O_2 diff. (ml./100 ml.)	6.5 ± 0.7	14.3 ± 2.5
n	5	15
"Central" volume (liters)	1.71 ± 0.32	3.49 ± 0.83
n	14	15
Appearance time (seconds)	11.9 ± 2.2	5.9 ± 1.3
n	14	15
Mean circ. time (seconds)	18.5 ± 3.1	9.4 ± 2.0
n	14	15

* Mean maximal O_2 intake determined previously on same group: 3.15 ± 0.31 L./min.

20 to 29 (4.15 ± 0.36 liters per minute) (16), and that of Slonim, Gillespie, and Harold (4.05 ± 0.39) (17) for 65 young trained men, were presumably determined to some extent by the high degree of physical training their subjects had reached (18). Buskirk and Taylor's comparable figure for a group of young trained subjects was 3.95 ± 0.43.

In order to render the test as reliable as possible, rigid criteria for determining the point at which maximal oxygen intake is reached must be established. If this is done, the test is quite repeatable (Table I) and a given value characterizes an individual for several months at least.

The effect of age on the maximal oxygen intake can be seen in Table II. At the initial exercise level the oxygen intake is the same for the first three age groups but the *maximal* intake values for all four age groups show a decrease with age. These results are comparable to those of Robinson (3). It is clear, therefore, that the age trend must be taken into account if the test is used for functional evaluation of human subjects.

In Table III it is noted that ventilation reaches its highest value at the maximal oxygen intake level. There is no significant difference in the ventilation at the maximal and the postmaximal level in a given age group. With increasing age the level of ventilation at maximal workloads tends to decline. Oxygen removal rates decline with increasing workloads in the first three age groups. In the oldest group, the oxygen removal rate ap-

TABLE V

Mean values for O_2 content, O_2 tension, per cent saturation, pH and hematocrit from one arterial and two venous sites at rest and during exercise at maximal oxygen intake level

Site	State	O_2 content *ml./100 ml.*	O_2 tension *mm. Hg*	Per cent sat.	pH	Hct. *per cent*
Brachial artery	Resting	18.1 ± 1.4	87 ± 9	97.1 ± 3.1	7.40 ± 0.04	46.5 ± 3.8
	n	14	15	9	15	14
	Max. O_2	19.3 ± 1.9	88 ± 12	94.7 ± 2.6	7.19 ± 0.09	48.3 ± 3.4
	n	14	15	9	15	14
Brachial vein	Resting	9.3 ± 2.4	31 ± 7	52.6 ± 12.9	7.36 ± 0.03	43.8 ± 2.7
	n	8	8	8	8	8
	Max. O_2	4.5 ± 1.2	29 ± 8	24.9 ± 7.8	7.13 ± 0.04	48.3 ± 3.0
	n	6	6	6	6	6
Femoral vein	Resting	6.6 ± 2.5	30 ± 11	37.9 ± 16.2	7.35 ± 0.07	45.3 ± 2.6
	n	8	8	8	8	7
	Max. O_2	3.9 ± 1.3	30 ± 7	19.9 ± 6.8	7.11 ± 0.05	48.2 ± 3.2
	n	8	8	8	7	8

TABLE VI

Values for cardiac output and A V O_2 difference in six normal subjects at the maximal oxygen intake and at a higher workload

	Maximal oxygen intake level			Beyond maximal oxygen intake level		
	O_2 intake	Cardiac output	AV O_2 diff.	O_2 intake	Cardiac output	AV O_2 diff.
J. B.	2.56	20.6	12.4	2.42	15.6	15.5
L. C.	3.23	25.9	12.5	3.03	21.1	14.4
A. G.	2.89	15.6	18.5	2.90	14.7	19.7
F. H.	3.01	23.2	13.0	2.96	17.1	17.3
C. Mc.	3.08	26.3	11.7	3.05	25.8	11.8
J. W.	2.47	14.1	17.5	2.52	15.1	16.7
Mean	2.87	21.0	14.3	2.81	18.2	15.9
S.D.	0.31	4.7	2.7	0.34	4.1	2.5

pears to remain constant with increasing workloads.

The primary purpose of the study was not, however, merely to re-examine previously established characteristics of maximal oxygen intake but, rather, to inquire into its physiological meaning. It is obvious that, in terms of the Fick equation, the main circulatory determinants must be cardiac output on the one hand, and AV oxygen difference on the other (19). The relative role played by each of these factors in determining the maximal oxygen intake is seen in Table IV. Oxygen intake increased 9.5 times from the resting state to the workload producing the maximal figure; the corresponding figure for cardiac output is 4.3. Proportionately, pulse rate and stroke volume increased equally—about two times. At the time the cardiac output reached its maximal level, the "central" blood volume, as calculated by the Hamilton method, had doubled. The appearance time of the dye and the mean circulation time had decreased by one-half.

On the face of all this, one might assume with Bainbridge (13) that cardiac capacity is the primary determinant of maximal oxygen intake. Such an assumption, however, ignores the fact that the AV oxygen difference makes a contribution of its own to oxygen intake. Simple increase in cardiac output by 4.3 times, AV oxygen difference remaining at 6.5 ml. per 100 ml., would increase oxygen intake from 340 ml. (resting), to no more than 1,500 ml. Widening of the AV oxygen difference by 2.2 times (from 6.5 to 14.3 ml. per 100 ml.), however, permits oxygen intake to exceed 3 liters under the same conditions. Were it not for the ability of the organism to widen its AV oxygen difference, cardiac output would have to increase nearly 10 times to supply 3,200 ml. of oxygen per minute to the tissues. Our mean value for AV oxygen difference during heavy work is in close agreement with that found by Asmussen and Nielsen (20), and Freedman, Snider, Brostoff, Kimelblot, and Katz (21), but higher than that found by Christensen (22).

The magnitude of the maximal AV oxygen difference is determined by the maximal level

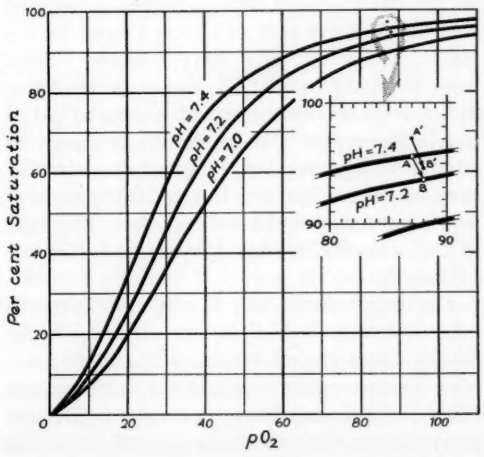

FIG. 2. OXYGEN DISSOCIATION CURVES AT THREE LEVELS OF pH (33)

Insert shows A and B (open circles), representing values for per cent saturation corresponding to two observed values for arterial pO_2 (87 mm. Hg at rest, and 88 mm. Hg during heavy work). A' and B' represent per cent saturation, calculated from Van Slyke determinations, for the same two pO_2 values. The fall in per cent saturation is about 2 per cent in each case.

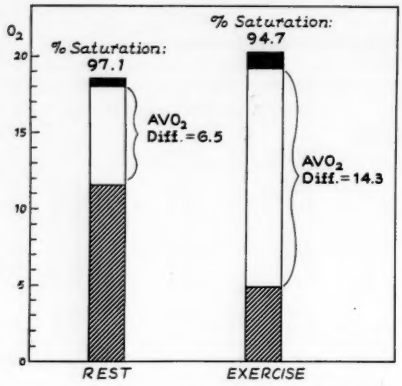

FIG. 3. THE AV OXYGEN DIFFERENCE AT REST AND DURING EXERCISE

Total height of each column represents oxygen capacity. Black areas at top are unoxygenated hemoglobin. Shaded areas at bottom represent mixed venous oxygen content.

reached by the oxygen content of arterial blood on the one hand, and the lowest level reached by the oxygen content of mixed venous blood on the other. In Table V it is noted that there is a 1.2 ml. per 100 ml. rise in arterial oxygen content and a slight fall in per cent saturation (about 2.4 per cent) between rest and heavy exercise. These items are easily understood when it is noted that the exercise also induces a fall in arterial pH, a rise in hematocrit, and no significant change in arterial oxygen tension (Figure 2). In any event, the small increases in arterial oxygen content induced by exercise can contribute very little to the widening of the AV oxygen difference (Figure 3).

The magnitude of the AV oxygen difference is influenced most markedly by encroachment on the mixed venous oxygen content (23). Mixed venous oxygen content (calculated from oxygen intake, cardiac output, and arterial oxygen content) decreased from 11.6 ml. per 100 ml. at rest to 5.0 ml. per 100 ml. during exercise at the maximal oxygen intake level. This encroachment on the mixed venous oxygen content is made possible by two mechanisms: One is the extent to which blood perfusing working tissue can surrender its oxygen; the other is the shunting of blood away from inactive areas as noted by Donald, Bishop, and Wade (24). The lowest level to which the oxygen content of blood flowing through strenuously working muscle may fall is not known but it seems unlikely that it ever approaches zero in normal subjects (25). An approach to these problems was made in the present study by comparing oxygen content and tension of blood from brachial and femoral veins during running. The brachial vein blood was drawn from the stationary arm. It is seen in Table V that heavy exercise reduced venous oxygen content to less than half its resting value in both samples, the femoral sample showing slightly greater reduction than the brachial. The oxygen content of the femoral venous sample (3.9 ± 1.3 ml. per 100 ml.) is probably near the lowest value that can be reached since it is made up primarily of blood from working muscle. But the drop in oxygen content of blood from the brachial vein of the stationary arm is almost as large (4.5 ± 1.2 ml. per 100 ml.) and is in keeping with the report of Donald, Bishop, and Wade (24) to the effect that during moderate exercise the oxygen content of venous blood from a resting extremity falls as low as that from one which is exercising. These findings, along with their observation that the volume of a resting arm is decreased during leg exercise, are most readily explained on the basis of physiological shunting of blood away from resting tissue during exercise. Such blood as continues to perfuse resting tissue may be so small in amount that it must surrender as much oxygen, relatively speaking, as blood perfusing exercising muscle. In any case, oxygen content of femoral and brachial venous blood during heavy exercise draws very close, as exercise proceeds, to the calculated mixed venous oxygen content (5.0 ml. per 100 ml.).

The most striking feature of the blood gas studies is the relative constancy of oxygen tension, both arterial and venous, during heavy exercise (Table V). In contrast, a decrease in arterial oxygen tension during low grades of exercise has been reported by other observers (26–28). Blount, McCord, and Anderson (29), however, found no change in arterial oxygen tension during similar exercise loads. Hickam, Pryor, Page, and Atwell (30), and Asmussen and Nielsen (31) noted a slight drop (about 2 per cent) in arterial oxygen saturation during heavy exercise but the first group (30) felt that the phenomenon might be due to pH changes and not to a decrease in ar-

terial oxygen tension. Our results support the latter view (Figure 2). Oxygen tension of brachial and femoral venous blood also tended to remain at or near the resting value even though oxygen content and per cent saturation are both markedly depressed during heavy work. Teleologically, it would seem that an adequate level of oxygen tension is vigorously protected so that an effective pressure gradient from capillary to tissue cell can be maintained. The decrease in pH and the increase in carbon dioxide tension during exercise combine to cause a temporary shift of the oxygen dissociation curve to the right in the systemic capillary, but the pH changes alone were not large enough to explain the magnitude of the shift as actually observed (Figure 3). The carbon dioxide tension of brachial venous blood increased from a mean value of 45 mm. Hg at rest to 65 mm. Hg at the maximal oxygen intake level of exercise; the corresponding figures for femoral venous blood were 50 mm. Hg and 65 mm. Hg. It may be, as suggested by Roughton (32), that increase in carbon dioxide tension tends, independently of change in pH, to cause a shift to the right of the oxygen dissociation curve. In any case, the shift makes it possible, as blood traverses the capillary, for oxygen to be given up more readily without disturbing the pressure gradient from capillary to cell. In the pulmonary capillary, the situation reverses itself, facilitating oxygenation of venous blood.

The maintenance of arterial oxygen tension during heavy exercise is adequate evidence against the possibility that pulmonary factors, ventilatory or diffusive, determine maximal oxygen intake. Nor did timed vitalometry, carried out at rest in some of the subjects, provide evidence of impairment of pulmonary function. One might well go further and hold that a definite decrease in arterial oxygen tension during heavy exercise, far from being a normal finding, is actually an indication of abnormal response to exercise.

In Table VI are presented the data on six normal men whose oxygen intake leveled or actually declined at workloads beyond that producing maximal intake. It is noted that as oxygen intake declined, the cardiac output also declined. The AV oxygen difference, however, appears to have increased still further. The possibility that the organism can increase AV oxygen difference even after cardiac output and oxygen intake have leveled off or are declining is open to several interpretations. It may mean that cardiac output is the more important determinant but the data at hand do not rule out the possibility that, in some subjects, further widening of the AV oxygen difference may permit oxygen intake to increase even after cardiac output has reached its peak.

In any event, the full meaning of maximal oxygen intake is in terms not merely of the ability of the heart mechanically to propel blood, but also of the ability of the tissues to extract oxygen from blood perfusing them. It seems unnecessary, therefore, to invoke the view that an important determinant of maximal intake is the inability of peripheral arteries and arterioles to take up all the blood pumped out by the vigorously contracting left ventricle (2, 16). The evidence now available suggests that maximal oxygen intake is a measure of cardiac capacity and the ability to increase the AV oxygen difference, not of the ability of the vascular bed to accommodate left ventricular output. With this in mind, the test may indeed have clinical usefulness in evaluating patients with certain types of cardiovascular disease.

CONCLUSIONS

1. The maximal oxygen intake is dependent on both cardiac output and AV oxygen difference. When the oxygen intake increased by 9.5 times from rest to the workload producing the maximal figure, the corresponding figure for cardiac output was 4.3 and for AV oxygen difference 2.2. The widening of the AV oxygen difference was due principally to diminution in mixed venous oxygen content.

2. There was no significant change in arterial oxygen tension from rest to heavy work; the slight decrease in oxygen saturation that was observed can be explained by the pH change of the blood and the resulting shift in the oxygen dissociation curve.

3. The venous oxygen tension showed no significant change, even though the venous oxygen content and saturation fell appreciably. The shift in the oxygen saturation curve to the right was due, in part, to decrease in pH, and (possibly) to an increased carbon dioxide tension as well.

The end result of the phenomenon is to maintain an adequate oxygen tension gradient from capillary to cell.

4. In ascertaining the physiological meaning of the maximal oxygen intake, the relative importance of cardiac capacity and increase in AV oxygen difference must be determined. It is probable that in the normal individual the ability to increase cardiac output is the more important of the two factors.

ACKNOWLEDGMENT

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MAINTENANCE OF POTASSIUM EXCRETION DESPITE REDUCTION OF GLOMERULAR FILTRATION DURING SODIUM DIURESIS

By DOUGLAS G. DAVIDSON, NORMAN G. LEVINSKY, AND ROBERT W. BERLINER
WITH THE TECHNICAL ASSISTANCE OF AGNES SCOTT PRESTON

(From the Laboratory of Kidney and Electrolyte Metabolism, National Heart Institute, Bethesda, Md.)

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It has been clearly established that mammalian renal tubules can secrete potassium. However, it has not been shown in mammals under physiologic conditions what proportion of the urinary potassium is derived from the filtered potassium and what proportion from secreted potassium. In experiments designed to study the various factors which influence potassium excretion it has sometimes been assumed that the reabsorption of the filtered potassium is essentially complete, and that these factors exert their effects on the secretory mechanism. However, evidence for the completeness of reabsorption of filtered potassium is limited and not conclusive.

The proposed tubular mechanism for the secretion of potassium is an ion exchange process in the distal tubule (1), where cellular potassium ions are exchanged for sodium ions derived from the glomerular filtrate. It is implicit in this mechanism that potassium secretion will depend in part on the availability of sodium ions for exchange. If the proximal reabsorption of filtered potassium is complete, the rate of excretion of potassium will be independent of the filtered load of potassium, providing an adequate distal load of sodium is maintained. To test this hypothesis, the glomerular filtration rate was reduced in one kidney and the rate of excretion of potassium from this kidney was compared to that of the control kidney, during both high and low rates of sodium excretion.

METHODS

Experiments were performed using trained unanesthetized female dogs weighing 18 to 22 Kg. During the experiments the dogs were standing, with light support from a sling. Multiple experiments have been done on eight dogs. In order to lower the glomerular filtration rate in one kidney, a small inflatable cufflike clamp was placed around the renal artery. In these experiments, the renal artery clamp was always placed around the right renal artery, because of the late branching of this artery as compared to the left renal artery. The clamp was seated around the unbranched renal artery as near as possible to its origin from the aorta. Separate urine collections from each kidney were made possible by a bladder splitting operation. The renal artery clamp and the method of separate urine collections from each kidney are described in detail in a previous communication from this laboratory[1] (2).

Inulin was used to measure the glomerular filtration rate, and was determined by the method of Walser, Davidson, and Orloff (3). The clearance of para-aminohippurate (PAH) was used as a measure of the effective renal plasma flow, and was determined by the method of Bratton and Marshall (4). Sodium and potassium in diluted plasma and urine were determined by flame photometry using lithium as an internal standard. Urine chloride was determined by potentiometric titration (5).

Constant rates of intravenous infusions were maintained by means of a Bowman infusion pump. In some of the experiments, mercurial diuretics, acetazolamide (Diamox®) or Na_2SO_4 were given, often along with infusions of sodium chloride or bicarbonate, to assure a high rate of sodium excretion when the glomerular filtration rate was reduced.

RESULTS

Reduction of the glomerular filtration rate during low rates of sodium excretion

In contrast to the rest of the experiments to be presented, Tables I and II and Figure 1 present the data from seven experiments in four different dogs in which no attempt was made to maintain a high rate of sodium excretion when the glomerular filtration rate was reduced. These were selected as representative of about 100 such experiments done for various purposes other than the study of potassium excretion. In some of these experiments, mannitol was added to the infusions to maintain urine flows adequate for clearance measurements when the glomerular filtration rate was reduced. The first two to four periods in each

[1] We are indebted to Dr. Edward Sharpe for the surgical preparations.

TABLE I

Effect of lowering the glomerular filtration rate without NaCl diuresis

Time (*min.*)	Urine flow (*ml./min.*) R	Urine flow (*ml./min.*) L	Inulin clearance (*ml./min.*) R	Inulin clearance (*ml./min.*) L	Excreted K (*μEq./min.*) R	Excreted K (*μEq./min.*) L	Excreted Na (*μEq./min.*) R	Excreted Na (*μEq./min.*) L	Glomerular filtration rate R/L	Excreted K R/L	Excreted Na R/L
0	Infusion: Inulin, 10 *mg./min.*; Pitressin®, 100 *mU/Kg./hr.* in 2.5% NaCl solution at 0.5 *ml./min.*										
30–60	0.10	0.12	41.5	48.0	38.1	44.0	7.1	10.8	0.87	0.87	0.66
62–93	0.10	0.10	44.4	44.0	34.3	36.0	3.7	5.1	1.01	0.95	0.73
96–123	0.12	0.13	46.3	46.5	42.6	44.1	4.2	5.7	1.00	0.97	0.74
125	Clamp inflated right renal artery										
187–208	0.10	0.23	40.4	54.2	37.1	64.4	2.6	33.0	0.75	0.58	0.08
210–234	0.10	0.24	42.4	55.9	36.7	73.0	1.9	24.6	0.76	0.50	0.08
235–257	0.12	0.28	44.7	60.3	43.6	89.0	2.7	37.0	0.74	0.49	0.07
260–290	0.08	0.25	36.2	56.3	26.8	76.0	1.8	39.2	0.64	0.35	0.05
292	Clamp partially deflated										
308–328	0.14	0.29	54.2	65.7	51.2	70.3	16.3	64.2	0.82	0.73	0.25
329–349	0.19	0.31	61.0	63.4	61.1	72.5	27.6	69.6	0.96	0.84	0.40
360–379	0.18	0.28	54.6	58.9	57.0	70.2	36.0	67.1	0.93	0.81	0.54
381–396	0.17	0.27	49.0	54.3	47.6	75.6	38.5	64.6	0.90	0.63	0.60

experiment were control periods, secured to compare the function of the two kidneys and especially to determine the ratio of the rates of excretion of potassium by the two kidneys. The absolute rate of potassium excretion from each kidney varies from period to period during the course of an experiment. No attempt has been made to define the many factors which affect this variation in potassium excretion. However, the assumption is made that these factors exert identical effects in both kidneys. Therefore, the effects of clamping the renal artery are interpreted in terms of changes in the ratio of the rates of excretion of potassium by the two kidneys, rather than in terms of changes in the absolute rate of potassium excretion by the "clamped" kidney.

Following the control periods, the glomerular filtration rate was reduced in the right kidney by inflating the clamp. Various levels of reduction of the glomerular filtration rate were secured in each experiment. Simultaneously with the reduction in glomerular filtration rate, there was a marked fall in the potassium excretion of the right relative to the left kidney. It is important to note that both the absolute and the relative rates of sodium excretion from the right kidney fell markedly during the periods of constriction, except for the last four periods in Table I in which

TABLE II

Effect of lowering glomerular filtration rate without NaCl diuresis

Time (*min.*)	Urine flow (*ml./min.*) R	Urine flow (*ml./min.*) L	Inulin clearance (*ml./min.*) R	Inulin clearance (*ml./min.*) L	Excreted K (*μEq./min.*) R	Excreted K (*μEq./min.*) L	Excreted Na (*μEq./min.*) R	Excreted Na (*μEq./min.*) L	Glomerular filtration rate R/L	Excreted K R/L	Excreted Na R/L
	Prime: 400 *mg.* inulin										
0	Sustaining infusion: Inulin, 10 *mg./min.*; mannitol, 2,400 *μOsm./min.* in water at 5 *ml./min.*										
40–50	1.92	2.14	36.4	36.4	61.3	64.8	118	158	1.0	0.95	0.75
51–56	2.30	2.44	36.0	35.4	80.6	83.2	140	167	1.02	0.97	0.84
57–64	2.30	2.37	35.0	35.4	93.6	91.6	134	157	0.99	1.02	0.85
67	Clamp inflated right renal artery										
118–125	1.97	4.31	26.1	38.8	53	118	76.3	288	0.67	0.45	0.27
128–133	2.18	4.84	29.2	39.6	60	127	50.9	335	0.74	0.47	0.15
134–141	1.94	4.65	24.0	37.0	47	127	36.6	318	0.65	0.37	0.12
145–151	2.38	4.48	26.8	35.6	63	130	66.8	308	0.75	0.49	0.22
152–160	2.45	4.44	29.2	36.4	69	132	60.9	278	0.80	0.52	0.22

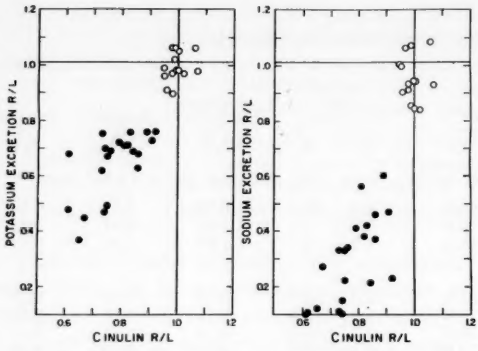

FIG. 1. MASS PLOT OF DATA FROM FIVE EXPERIMENTS IN WHICH A HIGH RATE OF SODIUM EXCRETION WAS NOT MAINTAINED

Open points represent control periods; solid points, periods in which the filtration rate in the right kidney was lowered.

the absolute rates of excretion of both sodium and potassium increased, although both remained low, relative to those on the control side. Figure 1 is a mass plot of the data from five experiments (not including those in Tables I and II) in four different dogs. In the left half of Figure 1, the ratio of the rates of potassium excretion, right to left kidney, is plotted against the ratio of the clearance of inulin. A decrease in the ratio of potassium excretion occurs at all levels of reduction of filtration rate. In the right half of Figure 1, the ratio of sodium excretion is similarly plotted. When filtration rate is lowered to any measurable degree, a marked fall in sodium excretion is evident.

In the interpretation of the results of these experiments, the absolute rate of sodium excretion has been considered to be indicative of the load of sodium to the distal tubule. In all of these experiments the fall in the sodium excretion of the right kidney, when compared to the left, exceeded the relative decrease in the clearance of inulin. The fall in the rate of potassium excretion of the right relative to the left kidney is interpreted as a consequence of the decrease in sodium available for exchange with potassium in the distal tubules, as manifested by the marked drop in sodium excretion. A fall in the ratio also could be effected by a decreased filtered load of potassium if the filtered potassium did contribute significantly to the excreted potassium. In addition, dropping out of nephron units during constriction of the renal artery could account for a fall in potassium excretion. However, the latter explanations are unlikely in light of the results of experiments performed during high rates of sodium excretion.

Reduction of the glomerular filtration rate during high rates of sodium excretion

These experiments were designed to assure adequate loads of sodium in the distal tubule despite reductions in the glomerular filtration rate. Table III gives the data from an experiment in which adequate sodium excretion was secured by the administration of a mercurial diuretic and the infusion of isotonic saline. During the control periods, the clearance of inulin and the excretion of potassium and sodium from each kidney were

TABLE III

Effect of reducing glomerular filtration rate during mercurial diuresis

Time (*min.*)	Urine flow (*ml./min.*) R	L	Inulin clearance (*ml./min.*) R	L	Excreted K (*μEq./min.*) R	L	Excreted Na (*μEq./min.*) R	L	Excreted Cl^- (*μEq./min.*) R	L	Plasma K (*mEq./L.*)	Filtered K (*μEq./min.*) R	L	Glomerular filtration rate R/L	Excreted K R/L	Excreted Na R/L
	Prime: Inulin, 700 *mg.*; Salyrgan®, 2 *ml.* I.V.															
	Sustaining infusion: Inulin, 12.5 *mg./min.*; NaCl, 1,800 *μEq./min.*; Salyrgan®, 0.012 *ml./min.*; in water at 12 *ml./min.*															
65–71	5.59	5.50	58.6	58.4	15.3	17.5	954	929	825	813	4.37	256	255	1.00	0.87	1.03
72–77	7.80	7.05	63.0	56.7	16.6	18.2	1,200	1,081	1,060	959	4.37	276	248	1.10	0.91	1.11
81–85	9.70	8.90	57.6	56.0	19.4	21.6	1,470	1,370	1,310	1,190	4.34	250	244	1.03	0.90	1.07
90	Clamp inflated right renal artery															
174–178	5.37	9.50	40.0	64.1	52.3	54.9	816	1,420	726	1,290	4.20	168	269	0.63	0.95	0.57
180–185	6.0	10.10	42.6	62.5	55.8	59.1	911	1,520	820	1,380	4.23	180	264	0.68	0.94	0.60
188–192	8.26	9.75	52.0	63.6	62.6	61.3	1,270	1,500	1,130	1,340	4.36	226	278	0.82	1.03	0.85
193–198	6.41	8.10	46.0	60.2	57.5	59.0	1,019	1,340	913	1,160	4.45	205	268	0.76	0.98	0.76
200–205	6.60	8.43	46.0	64.5	54.1	58.1	1,028	1,340	925	1,190	4.48	206	289	0.71	0.93	0.77

close to equal. The glomerular filtration rate in the right kidney was then reduced by compression of the renal artery. The elapsed time between the control periods and the first experimental period represents the time necessary to achieve a stable reduction of the glomerular filtration rate. During this time, the full effect of the mercurial and sodium chloride infusion on potassium excretion also becomes manifest, as is shown by the increase in the absolute rate of potassium excretion. The filtration rate was reduced by 19 to 37 per cent in the right kidney. Despite a reduced filtered load of potassium, the potassium excretion of the right kidney was not decreased relative to that of the left kidney. The fall in the rate of sodium excretion from the experimental kidney was approximately proportional to the reduction in the glomerular filtration rate. However, despite the lowered filtration rate, the absolute rate of sodium excretion remained high. The data from a similar experiment in a different dog are plotted in Figure 2. Again, a mercurial diuretic and an infusion of isotonic sodium chloride assured adequate sodium excretion. The solid bars represent the data from the experimental kidney, and the stippled bars, the data from the control kidney. The total length of each bar represents the filtered load of potassium in each kidney, and the distance of the bars below the zero line, the rates of excretion of potassium. In the three control periods

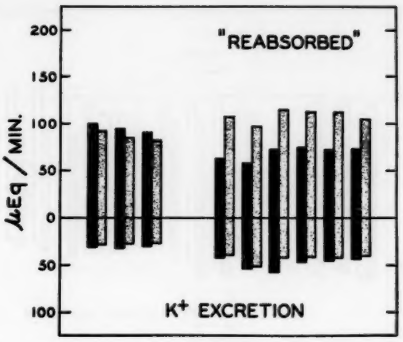

FIG. 2. POTASSIUM EXCRETION DURING INFUSION OF A MERCURIAL DIURETIC AND NaCl

Each pair of bars (solid, control kidney; stippled, experimental kidney) represents a clearance period. The first three pairs are control periods. The total length of each bar represents the filtered load of potassium; the distance of each bar below the zero line, the rate of excretion of potassium.

shown on the left in the figure, the rate of potassium excretion by the experimental kidney is slightly greater than that of the control kidney. During the periods of compression of the renal artery shown on the right in the figure, despite the marked decrease in the filtered load of potassium of the experimental kidney relative to the control kidney, the rate of excretion by the experimental kidney remained slightly higher than that of the

TABLE IV

Effect of reducing glomerular filtration rate during $NaHCO_3$–Diamox® diuresis

Time (*min.*)	Urine flow (*ml./min.*) R	L	Inulin clearance (*ml./min.*) R	L	PAH clearance (*ml./min.*) R	L	Excreted K (*μEq./min.*) R	L	Excreted Na (*μEq./min.*) R	L	Plasma K (*mEq./L.*)	Filtered K (*μEq./min.*) R	L	Glomerular filtration rate R/L	Excreted K R/L	Excreted Na R/L
	Prime: Inulin, 500 *mg.*; Diamox®, 250 *mg.*															
0	Sustaining infusion: Inulin, 10 *mg./min.*; PAH, 6 *mg./min.*; $NaHCO_3$, 1,800 *μEq./min.*; Diamox®, 1.5 *mg./min.*; in water at 12 *ml./min.*															
39–45	5.90	6.25	46.0	47.5	179	190	121	137	1,008	1,232	4.46	205	212	0.97	0.88	0.82
47–52	6.34	6.89	45.0	49.0	216	197	120	139	1,016	1,216	4.34	195	213	0.92	0.87	0.84
53–57	7.86	8.25	42.5	45.0	199	212	136	147	1,180	1,428	3.97	169	179	0.94	0.93	0.83
58–62	8.28	8.75	43.0	46.0	208	210	146	162	1,216	1,476	3.50	151	161	0.93	0.90	0.82
65	Clamp inflated right renal artery															
105–108	4.76	9.31	29.0	38.1	182	215	214	246	652	1,252	3.67	106	140	0.76	0.87	0.52
110–113	5.06	8.14	31.0	41.0	180	208	229	242	632	1,196	3.68	114	151	0.76	0.95	0.53
115–118	5.14	8.56	30.8	44.0	179	222	226	261	652	1,264	3.69	114	162	0.70	0.87	0.51
120–124	4.50	7.45	28.1	41.0	155	195	209	224	596	1,148	3.70	104	152	0.68	0.93	0.52
125–130	5.50	8.37	33.6	46.6	152	184	215	243	744	1,264	3.76	126	175	0.72	0.88	0.59
132–136	5.29	8.19	34.1	41.6	143	172	201	236	712	1,176	3.78	129	157	0.82	0.85	0.61
139–143	5.36	7.95	30.6	42.5	145	172	199	222	732	1,148	3.79	116	162	0.72	0.90	0.64

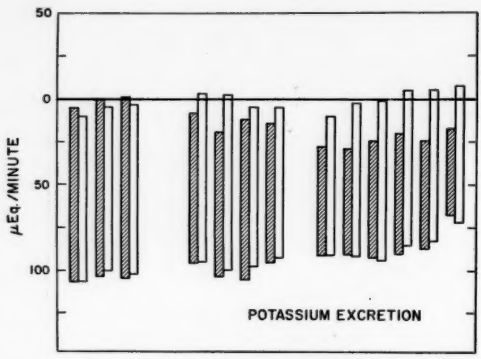

FIG. 3. POTASSIUM EXCRETION DURING INFUSION OF DIAMOX® AND $NaHCO_3$

Each pair of bars (cross-hatched, control kidney; open, experimental kidney) represents a clearance period. The first three pairs are control periods; the other two groups of bars are data obtained during two different settings of the arterial clamp. The total length of each bar represents the filtered load of potassium; the distance of the top of the bar from the zero line, net secretion of potassium; the distance of the bottom of the bar from the zero line, the rate of potassium excretion.

control kidney. The greater rate of potassium excretion from the experimental kidney during the third period of compression probably represents a dead space error due to a rising urine flow. The length of the bars above the zero line during the periods of compression of the artery shows the variation in reabsorption of potassium which would be needed to maintain an equal rate of potassium excretion, if the filtered potassium did contribute significantly to the excreted potassium. This would require a decrease in the proportion of the filtered load of potassium which is reabsorbed, when the filtered load is decreased by clamping the renal artery.

In the experiments in which mercurial diuretics were used to produce a high rate of sodium excretion, the excreted potassium was only a small portion of the amount filtered. The same independence of excretion rate from filtered load was found when potassium excretion was equal to or greater than the amount filtered. A high rate of excretion of sodium and potassium was made possible by the administration of Diamox®, a carbonic-anhydrase inhibitor. A sustaining infusion of sodium bicarbonate was given to prevent depletion of bicarbonate stores. The data from an experiment in which the rate of potassium excretion exceeded the filtered load are given in Table IV. Control periods were secured to establish the rates of potassium excretion by the two kidneys. The glomerular filtration rate was then reduced by 12 to 27 per cent with essentially no change in the relative rates of potassium excretion by the two kidneys. As in the two previous experiments, the absolute rate of sodium excretion remained high. The data from a similar experiment are plotted in Figure 3. The design of this figure is similar to that of Figure 2. The cross-hatched bars represent data from the experimental kidney, and the open bars, data from the control kidney. The length of the bars represents the rate of filtration of potassium; the distance of the bottom of the bars from the zero line, the rate of potassium excretion; the distance of the top of the bars below the zero line, the net secretion of potassium. The rate of excretion of potassium was high and in some periods exceeded the filtered load. Following the control periods the filtered load of potassium in the experimental kidney relative to the control kidney was decreased by lowering the filtration rate. This is shown by the shorter length of the shaded bars. In this experiment, as in the previous experiments, the rates of potassium excretion by the two kidneys remained essentially equal despite marked differences in the filtered loads.

A similar independence of excreted potassium from the filtered load of potassium was found in experiments in which sodium sulfate was used to promote a high rate of sodium excretion. Figure 4 summarizes the experimental data from multiple experiments in five dogs. The three materials used to produce an adequately high rate of sodium excretion were a mercurial diuretic (Salyrgan®), sodium sulfate, and Diamox®. The top half of the figure represents the data from three experiments in one dog; the bottom half of the figure, the data from experiments in four different dogs. Each point represents one clearance period. In the left half of the figure the ratio of the rates of potassium excretion, right to left kidney, is plotted against the ratio of the clearances of inulin. The right half of the figure is a similar plot of the sodium excretion. The ratio of the rates of potassium excretion from the two kidneys remained essentially unchanged with reductions

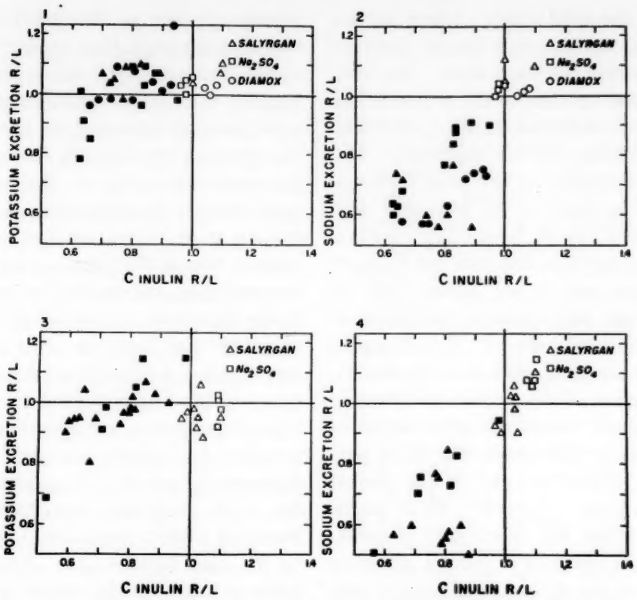

FIG. 4. MASS PLOT OF DATA FROM EXPERIMENTS IN WHICH SODIUM EXCRETION WAS MAINTAINED AT HIGH RATES

The upper half of the figure represents three experiments in one dog; the lower half, one experiment in each of four different dogs. The open points represent control clearance periods; the solid points, periods in which the filtration rate in the right kidney was lowered. (Two points in Chart 2 and six points in Chart 4, in which the ratio of sodium excretion, R/L, was less than 0.5, are not plotted.)

of the clearance of inulin of the experimental kidney to 60 to 65 per cent of that of the control kidney. The fall in the sodium excretion of the "clamped" kidney was proportional to or slightly greater than the reduction of the clearance of inulin. The results were independent of the agent used to produce a sodium diuresis.[2]

[2] In one dog only two levels of reduction of the glomerular filtration rate could be secured by inflation of the cuff; *i.e.*, 5 to 10 per cent and 50 to 53 per cent. At the 5 to 10 per cent level of reduction, potassium excretion was maintained. However, despite a marked sodium diuresis, when the filtration rate was reduced to 50 to 53 per cent, potassium excretion fell by approximately 50 per cent. The concentrations of Na, K, and inulin remained equal to that of the control kidney. It was suspected that the clamp, by error, had been placed so as to encircle two branches of the renal artery, and that with compression of the clamp, one branch was completely shut off and the other branch unaffected. To determine whether the entire kidney was being perfused when the clamp was set so as to reduce the glomerular filtration rate 50 per cent, fluorescein was injected rapidly intravenously. Following the appearance of the dye in the urine, the cuff was inflated with pressure sufficient to completely occlude the renal artery. The animal was sacrificed and the kidney immediately removed. A coronal section of the kidney was made and the distribution of the dye was determined by placing the kidney in ultraviolet light. Approximately one-half of the cut section was fluorescent and the other half was not; the dye was distributed only in one sagittal half of the kidney. In this particular preparation the mechanism of reduction of the glomerular filtration rate would seem to have been a dropping out of nephron units due to the complete occlusion of one branch of the renal artery.

DISCUSSION

In these experiments the glomerular filtration rate of one kidney has been reduced by mechani-

cal compression of the renal artery. There are two mechanisms which could account for the observed decrease in glomerular filtration rate. The first, and the more tenable in view of the experimental results, is a general reduction of the glomerular filtration rate involving all the nephrons. The second possible mechanism is complete interruption of blood flow to some of the glomeruli with consequent dropping out of functioning nephron units. The glomerular filtration rate has been reduced up to 40 per cent in one kidney with no decrease in the rate of potassium excretion as compared to the control kidney. If a dropping out of functioning nephron units were the mechanism for the reduction of the glomerular filtration rate, the experimental results could be explained only if it were further postulated that these particular nephrons did not take part in the normal excretion of potassium. However, these postulates would not explain the dependence of potassium excretion on a high rate of sodium excretion during reduction of the glomerular filtration rate. Evidence against dropping out of nephron units was also found by Thompson, Barrett, and Pitts (6) who reduced the glomerular filtration rate in dogs by inflating a balloon in the abdominal aorta and found no change in the maximum tubular reabsorption of glucose even when the glomerular filtration rate was significantly reduced. In some of the present experiments, during sodium diuresis when the reduction in filtration rate exceeded 40 per cent, there was a decreased rate of potassium excretion as compared to the control kidney. At these levels of reduction of filtration rate a decrease in the rate of potassium excretion may be due either to a loss of functioning nephron units or to an inadequate load of sodium to the exchange mechanism in some of the nephrons.

The independence of potassium excretion from the filtered load of potassium during sodium diuresis cannot reasonably be explained by any changes in potassium reabsorption. If the filtered potassium did contribute to the excreted potassium, the proximal tubules would have to reabsorb a smaller per cent of the filtered load during the periods of compression of the artery. A reabsorptive mechanism of this sort, which decreased the per cent of the load reabsorbed when the load presented to it is decreased, would show behavior exactly opposite to all other reabsorptive mechanisms which have been studied. Moreover, this would necessitate the assumption that the reabsorptive mechanism could modify its activity so as to leave unreabsorbed the same absolute amount of potassium even though the concentration of the potassium remaining in the lumen varied even more than the changing filtered load. This would require some means for the tubule to sense the volume flow in the lumen, integrate this with the concentration, and reabsorb potassium accordingly. In the experiments in which the rate of potassium excretion was high, in some periods there was net secretion by the experimental kidney and net reabsorption by the control kidney. Similarly in these experiments, variation in the proximal reabsorption of potassium is an unlikely mechanism for maintaining equality of potassium excretion. It has been suggested previously that the major source of urinary potassium is potassium secreted by the renal tubules (7). The ability of the tubules to reabsorb the entire filtered load of potassium has been shown in many studies in which potassium virtually disappeared from the urine of human subjects or animals depleted of potassium. The results of the present experiments are in complete accord with the hypothesis that reabsorption of filtered potassium is essentially complete and that the source of urinary potassium is secreted potassium. This seems to be the case during both low and high rates of potassium excretion.

In this study independence of potassium excretion from filtered potassium could be demonstrated only during sodium diuresis. No differences pertinent to the interpretation of these experiments were found among the various diuretics used. In the experiments in which no diuretic agent was administered and the absolute rate of sodium excretion fell to low levels, there was a marked fall in the relative rate of potassium excretion when the glomerular filtration rate was reduced. The fall in the rate of potassium excretion is interpreted as due to a decrease in the amount of sodium available for exchange with potassium. The dependence of potassium excretion on sodium lends strong support to the thesis that potassium is secreted in the distal tubule by the exchange of potassium for sodium (1).

SUMMARY

The excretion of potassium was studied in the dog during reduction of the glomerular filtration rate by means of an inflatable cuff placed around one renal artery. In experiments in which no attempt was made to maintain high rates of sodium excretion, potassium excretion fell with the decrease in the glomerular filtration rate. When high rates of sodium excretion were maintained by administration of mercurials, sodium sulfate, or Diamox®, the rate of potassium excretion was maintained in spite of reductions of the glomerular filtration rate by up to 40 per cent. This was true both at low and high rates of potassium excretion. The results support the interpretation that reabsorption of filtered potassium is essentially complete, and that the urinary potassium is derived from secreted potassium. The dependence of potassium excretion on sodium excretion provides evidence that the secretion of potassium occurs by an exchange of potassium for sodium in the distal tubule.

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THE *IN VITRO* BINDING OF COBALT^{60} LABELED VITAMIN B_{12} BY NORMAL AND LEUKEMIC SERA

By AARON MILLER with the technical assistance of JOHN F. SULLIVAN

(From the Radioisotope and Medical Services, Boston Veterans Administration Hospital, and The Department of Medicine, Boston University School of Medicine, Boston, Mass.)

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In chronic myelogenous leukemia both the serum concentration of vitamin B_{12} and the capacity of the serum to bind added vitamin B_{12} are markedly increased (1–3), suggesting an abnormality of the serum proteins in this disease. *In vitro* measurements of vitamin B_{12} binding capacity employing a microbiological assay method have not demonstrated a qualitative difference between normal and leukemic sera in respect to the binding of added vitamin B_{12} by the various serum protein fractions (1, 4). In the present study an equilibrium dialysis technique using cobalt^{60} labeled vitamin B_{12} has been employed to measure the binding capacity of normal and leukemic sera for added vitamin B_{12} and to identify the protein fractions which bind the added vitamin B_{12}.

METHODS

A. Experimental plan

Venous blood samples were obtained from patients in the fasting state. After two to three hours at room temperature the serum was separated under sterile conditions and stored at $-20°$ C. until tested. The serum concentration of vitamin B_{12}, the capacity of the serum to bind added cobalt^{60} labeled vitamin B_{12} and the distribution of the added vitamin B_{12} among the serum proteins were determined. Studies were carried out on 13 patients with chronic myelogenous leukemia, 8 patients with other types of leukemias, 8 patients with nonmalignant granulocytic leukocytoses, 2 patients with polycythemia vera and 22 control subjects, the latter group being composed of healthy laboratory personnel or ambulatory hospitalized patients suffering from functional disorders.

B. Analytical procedures

Serum vitamin B_{12} concentration. "Total" vitamin B_{12} concentration of the serum was determined by the modification of Lear, Harris, Castle, and Fleming (5) of the *Euglena gracilis* method of Ross (6). "Free" vitamin B_{12}, measured on occasional serum samples from normal subjects and patients with chronic myelogenous leukemia, constituted 15 per cent or less of the total serum vitamin B_{12}.

Serum vitamin binding capacity. Following the addition of a known excess quantity of radioactive vitamin B_{12} to serum, the degree of binding of the added vitamin B_{12} could be determined by a modification of the equilibrium dialysis method as described by Klotz, Walker, and Pivan (7). To duplicate or triplicate 2 ml. samples of serum was added 2 ml. of a solution containing 0.40 mμg. of Co^{60} vitamin B_{12} and 5 ml. of 1/15 M phosphate buffer (pH 7.3). This mixture was placed in a Visking[1] cellulose dialysis sac at 22° C. A protein-free blank prepared in triplicate with phosphate buffer in place of serum was run simultaneously. Each dialysis bag was placed into 3 by 17 cm. test tubes and the amount of radioactivity in each sample determined by means of a plastic scintillation well counter. After one hour incubation at 22° C., the bags were dialyzed against 250 ml. of 1/15 M phosphate buffer, pH 7.3. Initially, the dialysis was carried on for 24 hours at 22° C. with constant agitation but subsequently this was performed for 48 hours at 4° C. without agitation. Equilibrium was attained in both situations, and similar values for vitamin B_{12} binding were obtained under both experimental conditions. At the conclusion of dialysis each bag was rinsed externally in running water for one-half to one minute after which its radioactivity was determined in the plastic scintillation well counter. The bags were then slit in two, emptied of their contents, washed for one-half to one minute in running water and recounted for the measurement of the residual radioactivity bound to the bag. The unsaturated vitamin B_{12} binding capacity of the serum (UBBC) was determined from the following calculations:

1. Fraction of added $Co^{60}B_{12}$ bound per ml.

$$= \frac{(a)\ Co^{60}B_{12} \text{ in bag postdialysis (cpm)} - Co^{60}B_{12} \text{ bound to bag (cpm)}}{Co^{60}B_{12} \text{ added predialysis (cpm)} \times \text{ml. serum}} - \text{mean } Co^{60}B_{12} \text{ in blank calculated as in } (a)$$

2. UBBC, mμg./ml. = $Co^{60}B_{12}$ added predialysis (mμg.) × calculation *1*.

[1] Secured from Visking Corporation, Chicago, Illinois.

No correction for Donnan's equilibrium was needed as vitamin B_{12} is essentially un-ionized (8). The total vitamin B_{12} binding capacity of the serum (TBBC) was obtained from the sum of the serum vitamin B_{12} concentration and the UBBC.

Technical considerations. Two ml. of a 1:50 dilution of Co^{60} vitamin B_{12} of specific activity 0.97 μc./1.00 μg. were used in each determination of the UBBC unless otherwise stated. This was equivalent to 22 to 23,000 cpm under the counting conditions used. Radioactivity measurements were made in a large plastic scintillation well counter (well, 3.7 cm. in diameter and 10 cm. in depth) with other physical characteristics as previously described (9). A Co^{60} standard was counted each morning to correct for daily variations in the counter. The geometry of counting in pre- and postdialysis samples was constant. Samples were counted sufficiently long to give a counting error of less than 2 per cent except for the counting of empty dialysis bags where less than 5 to 7 per cent counting error was obtained.

The concentration of the stock Co^{60} vitamin B_{12} used for the measurement of the UBBC was determined by the Euglena gracilis assay. Due to the known photolability of the cyano group of the vitamin B_{12} molecule, aliquots of the stock $Co^{60}B_{12}$ solution were exposed to a fluorescent light source at 4° C. for a 12 day period. The binding of the Co^{60} vitamin B_{12} solution exposed to light was 80 per cent greater than a similar aliquot of the vitamin B_{12} solution protected from light. For this reason, the Co^{60} vitamin B_{12} was shielded from any source of light during all phases of the binding measurement. Serum, diluted 1 to 4 with 1/15 M phosphate buffer, pH 7.3, had a similar binding capacity as that of undiluted serum. Increasing or decreasing the amount of Co^{60} vitamin B_{12} over a fourfold range, slightly increased, or did not affect, the amount of vitamin B_{12} bound in both the normal and leukemic sera (Table I).

Experiments designed to evaluate the effect of temperature on vitamin B_{12} binding indicated that similar amounts of Co^{60} vitamin B_{12} were bound by serum at 4° C. and 22° C. No difference in binding was noted between aliquots of serum dialyzed at 4° C. and 22° C. The binding of radioactive material to the dialysis bag was influenced by the dialysis temperature however, 4 to 8 per cent of the Co^{60} vitamin B_{12} becoming bound at 22° C. as compared to 1 to 2 per cent at 4° C. The firmness of this bond is reflected by the fact that only 10 to 20 per cent of the bag radioactivity disappeared after 48 hours of dialysis. Dialysis against distilled water, phosphate buffer, pH 7 to 8, dextran solution, barbital buffer (pH 8.6) and saline had no effect on binding. Multiple successive freezing and thawing of serum did not affect binding capacity. The UBBC of serum stored at −20° C. remained stable over a six month period. In a patient with chronic myelogenous leukemia, no difference in vitamin B_{12} binding was found between serum collected, as described previously, and heparinized plasma separated from the cellular elements at 4° C. immediately after venipuncture. Serum with increased vitamin B_{12} binding showed better agreement between duplicate samples than those with normal binding. The vitamin B_{12} binding values showed a maximum difference in duplicate samples of 15 per cent in serum with binding greater than 4.00 mμg. per ml., 20 per cent with binding from 1.50 to 4.00 mμg. per ml., and 35 per cent in samples with binding less than 1.50 mμg. per ml. If sufficient serum was available, vitamin B_{12} binding was remeasured when the upper limits of precision were approached. The variation in the serum vitamin B_{12} concentration, the UBBC, and the TBBC of a normal subject over a two week interval of time is shown in Table II.

TABLE I

The relationship between the quantity of added vitamin B_{12} and the unsaturated vitamin B_{12} binding capacity of serum aliquots

	Amount of vit. B_{12} added per ml. serum mμg.	Unsaturated vit. B_{12} binding capacity mμg./ml.
Normal serum A	5	1.62
	10	1.86
	20	2.08
Normal serum B	4	1.33
	20	1.56
Myelogenous leukemic serum	10	7.20
	50	7.30

TABLE II

Vitamin B_{12} content and binding capacity of serum and the distribution of serum bound vitamin B_{12} as determined at different times in a normal subject

Subject	Serum B_{12} conc. mμg./ml.	UBBC* mμg./ml.	TBBC† mμg./ml.	Fraction of vitamin B_{12} bound to serum				
				Albumin %	α-1 globulin %	α-2 globulin %	β-globulin %	γ-globulin %
A. M.								
4/1/56	0.35	1.96	2.31	14	3	28	42	13
4/8/56	0.34	2.20	2.54	14	6	26	37	17
4/15/56	0.33	2.25	2.58	18	0	31	33	18

* UBBC refers to unsaturated vitamin B_{12} binding capacity.
† TBBC refers to total vitamin B_{12} binding capacity.

TABLE III

The vitamin B_{12} content and binding capacity of serum in 22 normal subjects as compared to 10 untreated cases of chronic myelogenous leukemia

	Serum vit. B_{12} concentration mμg./ml.		UBBC* mμg./ml.		TBBC† mμg./ml.	
	Range	Mean ± S.D.	Range	Mean ± S.D.	Range	Mean ± S.D.
Normal	0.27–1.27	0.64 ± 0.10	1.00–2.40	1.51 ± 0.56	1.32–2.81	2.23 ± 0.43
Chronic myelogenous leukemia	4.20–14.30	8.09 ± 4.55	4.75–14.25	8.87 ± 4.12	10.45–26.05	16.86 ± 7.78

* UBBC refers to unsaturated vitamin B_{12} binding capacity.
† TBBC refers to total vitamin B_{12} binding capacity.

Electrophoresis. One ml. of serum and 15 mμg. of Co^{60} vitamin B_{12} (in 0.5 ml.) were allowed to bind for one hour at 22° C. and then dialyzed at 4° C. for 48 hours against frequent changes of phosphate buffer, pH 7.3. Then, 0.12 ml. aliquots of the serum containing bound Co^{60} vitamin B_{12} were electrophoresed for 18 hours on 4 by 47 cm. Schleicher and Schuell paper No. 2043B, using veronal buffer pH 8.6 (ionic strength, 0.08) and a voltage of 110 to 114. At least four individual strips equivalent to 0.48 ml. of the serum-Co^{60} vitamin B_{12} mixture were run on each patient. Strips were then stained by a ninhydrin spray method. No loss of radioactivity occurred after staining the strips. This was in contrast to the bromphenol blue or amido black methods of staining where considerable losses of radioactivity were encountered. Under the conditions used, the albumin fraction usually moved 9 to 12 cm. from the origin while the gamma globulin moved slightly towards the anode (0.05 to 1.5 cm.). "Free" Co^{60} vitamin B_{12} (vitamin in saline solution) remained at the origin or moved slightly towards the anode (0.5 to 1 cm.). In serum which had not been dialyzed containing large amounts of unbound Co^{60} vitamin B_{12}, the free radiovitamin had a mobility similar to that in saline solution, occupying an area just adjacent to or overlapping that occupied by the gamma globulins. Each stained protein fraction was then sepa-

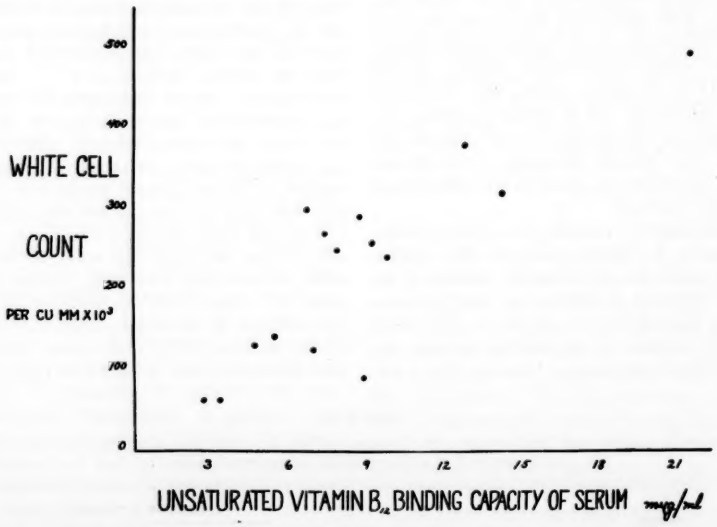

FIG. 1. CORRELATION OF THE WHITE CELL COUNT WITH THE UNSATURATED VITAMIN B_{12} BINDING CAPACITY IN 10 PATIENTS WITH CHRONIC MYELOGENOUS LEUKEMIA IN RELAPSE

The above points represent the white cell count and the unsaturated vitamin B_{12} binding capacity of a patient with chronic myelogenous leukemia in relapse. Some patients, studied at different times during one or more relapses, are represented by more than one point. The coefficient of correlation between the above two measurements was 0.88.

TABLE IV

Tabular summary of vitamin B_{12} data in treated and untreated cases of chronic myelogenous leukemia

Patient		WBC per cu. mm. $\times 10^3$	Serum B_{12} conc. *mμg./ml.*	UBBC *mμg./ml.*	TBBC *mμg./ml.*	Therapy
F. C.	10/4/56	3,814	14.30	12.75	27.05	X-ray to spleen
	10/29/56	280	11.53	1.70	13.23	
	12/7/56	623	3.30	2.75	6.05	
	4/5/57	5,005	6.50	20.00	26.50	X-ray to spleen
	4/15/57	1,506	5.50	14.40	19.90	
	4/20/57	749	6.20	8.99	15.19	
T. M.	6/13/56	2,109	7.83	9.22	17.05	P-32
	6/28/56	1,942	7.65			
	8/10/56	2,470	7.91	8.72	16.63	X-ray to spleen
	8/23/56	391	7.90	3.79	11.69	
	9/6/56	234	7.68	4.36	12.04	
	10/4/56	107	4.20	1.24	5.44	
	11/15/56	129	2.45	0.90	3.35	
J. C.	10/4/56	2,000	4.35	7.80	12.15	X-ray to spleen
	10/17/56	324	4.05	1.53	5.58	
	11/13/56	286	1.76	1.37	3.13	
	12/13/56	625	3.45	3.40	6.85	
	1/17/57	1,397	5.56	5.50	11.06	
	2/21/57	2,254		6.54		
S. M.	10/23/56	900	7.94	8.96	16.90	Myleran®
	12/13/56	145	3.80	2.90	6.70	
T. E.	9/11/56	766	2.25	5.00	7.25	5 weeks after incomplete response to X-ray, Myleran® begun
	11/15/56	59	2.19	2.18	4.37	
G. L.		185	0.94	1.40	2.34	1 yr. following treatment with Myleran®
S. D.		100	1.70	0.90	2.60	6 months following X-ray to spleen
S. B.		1,201	4.20	7.00	11.20	
A. R.		1,311	5.70	4.75	10.45	
R. L.		2,200	4.65	7.45	12.10	
T. W.		3,000	14.00	6.70	20.70	
T. K.		3,212	8.90	14.25	23.15	
V. P.		1,920	9.00	9.85	18.85	

rated by cutting out each individual protein fraction of the electrophoretic strip. Each of the combined stained protein fractions was then counted in a sodium iodide well counter. As low counting rates were usually found, each fraction was counted for 10 minutes, with the background for each sample determined by 10 minute counting preceding and following the sample count. The chief binding protein fractions in normal sera (beta- and alpha-2 globulins) counted from three-fourths to one and one-half times background, whereas the other fractions counted 10 to 40 per cent above background. In sera with high UBBC's the chief binding protein counted from two to four times background. The binding in each protein fraction was expressed as (*a*) per cent Co^{60} vitamin B_{12} bound calculated by dividing the radioactivity found in each protein fraction by the total radioactivity in all fractions times 100, and as the (*b*) mμg. of Co^{60} vitamin B_{12} bound per ml. calculated from the UBBC and calculation (*a*). The variation in vitamin B_{12} binding by the serum protein fractions of a normal subject measured over a two week interval of time is shown in Table II.

RESULTS

Serum vitamin B_{12} concentration and binding capacity

Normals. In 22 normal subjects the mean serum vitamin B_{12} concentration was equal to 0.64 mμg. per ml. (range, 0.27 to 1.27 mμg per ml.), the mean UBBC equal to 1.51 mμg per ml. (range, 1.00 to 2.40 mμg. per ml.), and the mean TBBC equal to 2.23 mμg. per ml. (range, 1.32 to 2.81 mμg. per ml.) (Table III).

Chronic myelogenous leukemia. The average serum B_{12} concentration, UBBC, and TBBC were greatly increased over normal in 10 patients with active chronic myelogenous leukemia, the first by a factor of 12, the second by a factor of 6, and the third by a factor of 7 (Table III). An excellent correlation was found between the white blood cell count and the UBBC in patients in relapse, the

coefficient of correlation equal to 0.88 (Figure 1). A poorer correlation was found between the white blood cell count and the serum B_{12} concentration (a coefficient of correlation of 0.58). With successful treatment of the disease (X-ray or Myleran®), the UBBC fell rapidly towards normal, paralleling the decline in white blood cell count, this phenomena being noted as soon as five days after the onset of therapy. Restoration of normal UBBC levels occurred within two weeks after the onset of treatment in one patient (Patient J. C., Table IV). By contrast, the concentration of vitamin B_{12} in the serum did not begin to decline for as long as two to three weeks after the onset of treatment. Even in complete remission serum B_{12} levels remained elevated with the sole exception of Patient G. L. (Table IV). With relapse, the serum vitamin B_{12} level and the UBBC rose together.

Other leukemias. The serum vitamin B_{12} content, the UBBC, and TBBC were normal in three patients with chronic lymphocytic leukemia except for a slight increase in TBBC in one of the three (Table V). Two patients with chronic monocytic leukemia had elevated serum B_{12} levels, UBBC's, and TBBC's approaching or equalling that found in chronic myelogenous leukemia. In contrast to chronic myelogenous leukemia, a poor correlation existed between the white blood cell count and the UBBC. After a partial response to X-ray treatment, the serum B_{12} level fell to normal whereas the UBBC and TBBC were elevated (Patient T. D.). One of three patients with acute leukemia had a moderate increase in UBBC and TBBC associated with a normal serum B_{12} level. This patient had a dramatic clinical response to cortisone associated with a decrease in the UBBC and TBBC to normal.

Leukocytosis Eight patients with neutrophilic leukocytoses due to varying causes with or with-

TABLE V

Tabular summary of vitamin B_{12} data in cases other than chronic myelogenous leukemia

Diagnosis and clinical comments	WBC per cu. mm. $\times 10^2$	Serum B_{12} conc. $m\mu g./ml.$	UBBC $m\mu g./ml.$	TBBC $m\mu g./ml.$
Chronic lymphocytic leukemia				
T. B.	4,000	1.06	1.65	2.71
G. M.	610	0.64	1.12	1.76
M. G.	3,866	1.27	1.97	3.24
Chronic monocytic leukemia				
T. D. 9/19/56	486	6.00	9.00	15.00
11/15/56 (Partial response to X-ray treatment)	129	0.50	5.50	6.00
J. M.	32	1.90	5.20	7.10
Acute leukemia				
T. C.	154	0.23	1.74	1.97
B. M.	1,862	0.73	1.26	1.99
S. C. 4/20/57	250	0.26	3.97	4.23
4/29/57 (Good response to cortisone)	41	0.20	1.96	2.16
Leukocytoses				
C. C. Pulmonary tuberculosis and bacterial pneumonia	352	0.98	1.55	2.53
T. D. Polycythemia vera with leukemoid reaction	580	0.85	6.30	7.15
J. F. Epidermoid carcinoma of throat with metastases	322	3.73	5.60	9.33
J. E. Septicemia	484	0.42	3.86	4.28
T. C. Pulmonary tuberculosis with leukemoid reaction	300	0.40	2.79	3.19
J. A. Carcinoma of lung with secondary infection	300	0.50	2.70	3.20
S. H. Polycythemia vera, postoperative after lobectomy for carcinoma of lung	534	0.25	2.94	3.19
T. A. ? Myeloid metaplasia	692	0.74	6.15	6.89
Polycythemia vera				
S. M.	100	0.57	2.40	2.97
T. S.	144	1.96	1.48	3.44
Miscellaneous				
J. R. Chronic pancytopenia of ? etiology	28	0.27	2.75	3.02

TABLE VI

The binding of Co^{60} labeled vitamin B_{12} by the protein fractions of serum from normal subjects

Subject	Albumin		α-1 globulin		α-2 globulin		β-globulin		γ-globulin	
	Vit. B_{12} bound %	Vit. B_{12} bound mμg./ml.	Vit. B_{12} bound %	Vit. B_{12} bound mμg./ml.	Vit. B_{12} bound %	Vit. B_{12} bound mμg./ml.	Vit. B_{12} bound %	Vit. B_{12} bound mμg./ml.	Vit. B_{12} bound %	Vit. B_{12} bound mμg./ml.
T. M.	11	0.18	7	0.11	29	0.47	32	0.52	21	0.34
F. M.	5	0.06	8	0.10	32	0.40	38	0.48	17	0.21
J. A.	6	0.11	7	0.12	23	0.41	48	0.85	16	0.28
H. L.	7	0.09	6	0.08	18	0.26	49	0.61	20	0.25
J. C.	4	0.08	1	0.02	16	0.31	66	1.29	13	0.25
A. C.	8	0.09	3	0.03	29	0.31	43	0.46	17	0.18
A. M.	18	0.35	8	0.16	29	0.57	37	0.73	8	0.16
F. M.	8	0.18	0	0.00	31	0.68	44	0.97	17	0.37
S. M.	4	0.10	4	0.10	47	1.14	32	0.78	13	0.31
F. M.	13	0.21	0	0.00	33	0.54	40	0.65	14	0.23
T. D.	11	0.26	2	0.05	34	0.82	56	1.34	17	0.41
T. G.	11		5		19		41		24	
M. S.	3		4		17		57		19	
S. F.	6		2		32		45		15	
M. L.	7		4		31		48		10	
T. M.	8		5		38		33		16	
P. R.	16		6		16		41		21	
T. B.	12		6		24		45		13	
Range	3–18	0.06–0.35	0–9	0–0.12	16–47	0.26–1.14	32–66	0.46–1.34	10–24	0.16–0.41
Mean ± standard deviation	9±4	0.16±0.09	4±3	0.07±0.06	28±7	0.59±0.19	44±10	0.79±0.38	16±4	0.27±0.09

out leukemoid reactions were studied (Table V). The commonest finding was a normal serum vitamin B_{12} concentration and an elevated UBBC and TBBC (six of eight patients). One patient (J. F.) had an elevated serum vitamin B_{12} concentration, UBBC, and TBBC approximating those found in chronic myelogenous leukemia while the last patient had a normal serum B_{12} concentration, UBBC, and TBBC.

Other diseases. One patient with polycythemia vera had a normal serum vitamin B_{12} level and UBBC but a slightly increased TBBC while another patient with this disease had an elevation of vitamin B_{12} content and TBBC. A patient with chronic pancytopenia of unknown etiology had a normal serum vitamin B_{12} level with a slight increase in UBBC and TBBC.

Electrophoresis of serum

Normals. In 16 of 18 normal subjects the beta globulins bound the greatest per cent of the added Co^{60} vitamin B_{12}, average of 44 per cent, range, 32 to 66 per cent (Table VI). The alpha-2 globulins were also an important binding fraction containing an average of 28 per cent of the added Co^{60} vitamin B_{12}, with a range of 16 to 47 per cent, and were the chief binding proteins in the two other subjects. Smaller amounts of radioactivity were bound by the gamma globulin and albumin fractions, whereas binding by the alpha-1 globulins was negligible (average, 4 per cent; range, 0 to 9 per cent). The amount of vitamin B_{12} bound per protein fraction expressed as mμg. per ml. is also shown in Table VI.

Chronic myelogenous leukemia. In contrast to normal subjects the alpha-1 or alpha-2 globulins were the main binding protein fractions of the serum for the added Co^{60} vitamin B_{12} (Table VII). Thus, the alpha-1 globulins contained an average of 38 per cent of the added Co^{60} vitamin B_{12}, approximately nine times that of normal sera, and was the main binding protein in 5 of the 10 patients. The alpha-2 globulins bound an average of 34 per cent of the added Co^{60} vitamin B_{12}, approximately 20 per cent more than that of normal serum, and was the main binding protein in four patients. When expressed in mμg. per ml. the increase in Co^{60} vitamin B_{12} binding by the alpha-1 globulins was approximately 47 times greater than that of normal serum as compared to the sixfold increase over normal by the alpha-2 globulins. The percentage of added Co^{60} vitamin

TABLE VII

The binding of cobalt^{60} labeled vitamin B_{12} by the protein fractions of serum from patients with chronic myelogenous leukemia in relapse

Patient	Albumin		α-1 globulin		α-2 globulin		β-globulin		γ-globulin	
	Vit. B_{12} bound %	Vit. B_{12} bound mμg./ml.	Vit. B_{12} bound %	Vit. B_{12} bound mμg./ml.	Vit. B_{12} bound %	Vit. B_{12} bound mμg./ml.	Vit. B_{12} bound %	Vit. B_{12} bound mμg./ml.	Vit. B_{12} bound %	Vit. B_{12} bound mμg./ml.
T. M.	3	0.28	56	5.16	16	1.48	10	0.92	15	1.38
S. B.	7	0.49	25	1.75	24	1.68	31	2.17	13	0.91
A. R.	9	0.43	24	1.14	34	1.62	19	0.90	14	0.66
F. C.	3	0.38	56	7.14	29	3.70	10	1.28	2	0.26
S. M.	4	0.36	52	4.66	23	2.06	13	1.17	8	0.72
J. C.	4	0.31	30	2.34	33	2.57	23	1.79	10	0.78
R. L.	1	0.07	21	1.57	55	4.10	18	1.34	5	0.37
T. W.	0	0.00	42	2.81	42	2.81	10	0.67	6	0.40
T. K.	1	0.14	36	5.13	52	7.41	7	1.00	4	0.57
V. P.	4	0.39	39	3.84	33	3.25	20	1.97	4	0.39
Range	0–9	0.00–0.49	21–56	1.14–7.14	16–55	1.62–7.41	7–31	0.67–2.17	4–14	0.26–1.38
Mean ± standard deviation	4±2	0.29±0.18	38±13	3.55±2.29	34±12	3.07±2.04	16±7	1.32±0.66	8±5	0.64±0.32

B_{12} bound by the beta globulins was decreased to about one-third that found for normal serum while slighter decreases were also found for the gamma globulin and albumin fractions. Despite the decrease in the per cent of added Co^{60} vitamin B_{12} bound by these fractions, modest elevations in the mμg. of vitamin B_{12} bound per ml. of serum were found although these values overlapped those found in normal serum. With successful treatment, the serum protein binding pattern reverted towards that found in normal subjects and, with complete remission, a normal pattern of binding was found (Table VIII).

Other leukemias. The binding of Co^{60} vitamin B_{12} by the serum proteins of patients with chronic lymphocytic leukemia was similar to that of normal serum (Table IX). Elevated binding by both the alpha-1 and alpha-2 globulins was found in one patient with chronic monocytic leukemia, whereas in two other patients with chronic mono-

TABLE VIII

The effect of treatment on the binding of cobalt^{60} labeled vitamin B_{12} by the protein fractions of serum from patients with chronic myelogenous leukemia

Patient	Albumin		α-1 globulin		α-2 globulin		β-globulin		γ-globulin	
	Vit. B_{12} bound %	Vit. B_{12} bound mμg./ml.	Vit. B_{12} bound %	Vit. B_{12} bound mμg./ml.	Vit. B_{12} bound %	Vit. B_{12} bound mμg./ml.	Vit. B_{12} bound %	Vit. B_{12} bound mμg./ml.	Vit. B_{12} bound %	Vit. B_{12} bound mμg./ml.
J. C.										
10/4/56 Rx	4	0.31	30	2.34	33	2.57	23	1.79	10	0.78
10/17/56 (After Rx)	2	0.03	8	0.12	28	0.42	39	0.60	23	0.35
1/17/57 (Relapse)	4	0.22	11	0.61	42	2.31	27	1.49	16	0.88
2/21/57 (Relapse)	2	0.13	22	1.35	42	2.75	22	1.35	12	0.79
F. C.										
10/4/56 Rx	3	0.38	56	7.14	29	3.70	10	1.28	2	0.26
10/29/56 (After Rx)	11	0.19	27	0.46	22	0.37	26	0.44	14	0.24
12/7/56 (Relapse)	2	0.06	20	0.55	55	1.51	18	0.50	5	0.14
4/5/57 Rx	2	0.40	35	7.00	52	10.40	8	1.60	3	0.60
4/15/57 Rx	2	0.29	40	5.76	47	6.77	7	1.01	4	0.58
4/20/57	7	0.63	25	2.25	51	4.59	12	1.08	5	0.45
G. L. Remission	6	0.08	7	0.10	46	0.64	34	0.48	7	0.10

TABLE IX

The binding of cobalt^{60} labeled vitamin B_{12} by the protein fractions of serum from patients with varying types of leukemia and leukocytoses

Diagnosis	Albumin		α-1 globulin		α-2 globulin		β-globulin		γ-globulin	
	Vit. B_{12} bound %	Vit. B_{12} bound mμg./ml.	Vit. B_{12} bound %	Vit. B_{12} bound mμg./ml.	Vit. B_{12} bound %	Vit. B_{12} bound mμg./ml.	Vit. B_{12} bound %	Vit. B_{12} bound mμg./ml.	Vit. B_{12} bound %	Vit. B_{12} bound mμg./ml.
Chronic lymphatic leukemia										
T. B.	8	0.13	3	0.05	30	0.50	47	0.78	12	0.19
G. M.	7	0.14	2	0.04	31	0.61	45	0.89	15	0.30
Chronic monocytic leukemia										
J. M.	4	0.21	3	0.16	40	2.08	41	2.13	12	0.62
T. D. 11/15/56 (After Rx)	1	0.06	18	0.99	54	2.97	22	1.21	5	0.28
Acute leukemia										
T. C.	8	0.14	6	0.11	20	0.35	35	0.61	31	0.54
S. C. 4/20/57	6	0.24	3	0.12	39	1.55	39	1.55	13	0.52
4/29/57 (After Rx)	11	0.22	3	0.06	30	0.59	49	0.96	7	0.14
Leukocytoses										
J. F.	0	0.00	25	1.40	58	3.25	14	0.78	3	0.17
J. A.	7	0.19	5	0.14	18	0.49	45	1.22	25	0.68
S. H.	7	0.20	6	0.18	18	0.53	53	1.56	16	0.47

cytic leukemia and acute leukemia, increased binding by the alpha-2 globulins was found (Table IX).

Leukocytoses. Patient J. F., with the increase in serum vitamin B_{12} concentration, UBBC, and TBBC, also demonstrated increased binding of Co^{60} vitamin B_{12} by the alpha-1 and alpha-2 globulins (Table IX). Two other patients with slight increases in the UBBC and TBBC had normal patterns of Co^{60} vitamin B_{12} binding by the serum proteins.

DISCUSSION

Amounts of vitamin B_{12} bound by the sera of normal subjects as determined in this study differ greatly from those previously obtained using microbiological assay procedures (3, 4, 5). Such differences in results are not surprising in view of the different methods employed. The binding of vitamin B_{12} is defined physicochemically in a dialysis method, *i.e.*, by the ability of the free vitamin to traverse a cellulose membrane. This experimental system is not subject to the variables inherent in microbiological assay procedures.

A relatively constant amount of vitamin B_{12} was bound by normal and leukemic sera when increasing concentrations of Co^{60} vitamin B_{12} were allowed to bind with normal and leukemic sera before dialysis. A similar limit in the vitamin B_{12} binding has been reported using a dialysis system (10). Recently, others have reported different results using a dialysis method differing slightly from that used in this study (11). They found no limitation in the amount of vitamin B_{12} bound by normal serum when the concentration of Co^{60} vitamin B_{12} was progressively increased. No reason for these differences in results is apparent at present.

In normal sera, using microbiological methods, the alpha globulins have been found to be the chief binding proteins for added vitamin B_{12} (4). However, the beta globulins bound the greatest amount of added Co^{60} vitamin B_{12} in the present study. The alpha-2 globulins were next in vitamin B_{12} binding ability but only negligible amounts were bound by the alpha-1 globulins. In previous investigations, excess vitamin B_{12} was added to serum, the serum proteins fractionated by paper electrophoresis, and the vitamin B_{12} content of the isolated protein fractions then determined. The considerable vitamin B_{12} found in the beta globulin fraction was ascribed to "free" vitamin B_{12},

i.e., that available to *Euglena gracilis* without preliminary heating of the serum. However, we have found that a large fraction of the Co^{60} vitamin B_{12} "bound" by normal serum as determined by dialysis is measured as "free" by the *Euglena gracilis* assay (12). The vitamin B_{12} in human liver homogenates has been found to be "free" as measured by *Euglena gracilis* even though the vitamin B_{12} was associated with a protein which had a mobility similar to the beta globulins on electrophoresis (13). Actually, on electrophoresis "free" vitamin B_{12} (the vitamin in saline solution) moves with the endosmotic flow and not as a beta globulin. It seems apparent that the differences in our findings and those previously reported are due to the different methods employed for the determination of vitamin B_{12} binding. The interpretation of the *Euglena gracilis* method of determining serum vitamin B_{12} binding is difficult if the vitamin B_{12} moving with a protein on electrophoresis can be measured as "free."

It is of interest that the vitamin B_{12} content of normal serum is primarily bound to the alpha-1 and alpha-2 globulins (4), whereas added vitamin B_{12} was principally bound by the beta and alpha-2 globulins. The interpretation of these findings is not certain. It may be that the alpha-1 globulins are already saturated with vitamin B_{12} accounting for their inability to bind additional vitamin B_{12}, whereas the alpha-2 globulins are only partially saturated and still capable of binding added vitamin B_{12}. The ready binding of added vitamin B_{12} by the beta globulins could be due to an *in vitro* avidity of the protein for the vitamin having no significance *in vivo*. However, *in vivo* binding of vitamin B_{12} by the beta globulins cannot be ruled out. Small amounts of the serum vitamin B_{12} content were found bound to the beta globulins in some normal sera (4). A protein in human liver homogenates resembling a beta globulin on electrophoresis is also capable of binding vitamin B_{12} (13). The small amount of vitamin B_{12} bound to the beta globulins *in vivo* could result if its rate of serum turnover was rapid as compared to the vitamin bound to the alpha globulins.

The vitamin B_{12} concentration, UBBC, and TBBC were all greatly elevated in chronic myelogenous leukemia as has been noted by other workers (1–3). The increase in UBBC was a labile abnormality decreasing rapidly to normal with the onset of treatment in contrast to the gradual decline in serum vitamin B_{12} levels following treatment. Of the other leukemias studied, only the patients with chronic monocytic leukemia had elevations in vitamin B_{12} concentration, UBBC, and TBBC approaching that found in chronic myelogenous leukemia. Elevated vitamin B_{12} levels have also been reported in monocytic leukemia (3, 14), although no increase in binding capacity was found using the *Euglena gracilis* binding method (3). Similarities between the monocytic and myelogenous leukemic processes are suggested by these findings. Patients with granulocytic leukocytoses of varying etiologies usually had normal serum vitamin B_{12} levels with increased UBBC's and TBBC's although the increase in UBBC and TBBC was usually not in the range found in chronic myelogenous leukemia. Thus, serum vitamin B_{12} levels were more helpful in distinguishing these patients from those with chronic myelogenous leukemia. However, this was not uniformly so, and Mollin and Ross have reported increased serum vitamin B_{12} levels in two patients with chronic leukocytoses (2).

In contrast to the findings in normal sera, the alpha-1 and alpha-2 globulins were the proteins responsible for tremendous increase in the UBBC of chronic myelogenous leukemic sera. The increase in binding by the alpha-1 globulins was most striking as negligible amounts of vitamin B_{12} were bound by this protein in normal serum. Similar increased binding of added vitamin B_{12} by the alpha-1 and alpha-2 globulins of myelogenous leukemic sera had been found, using microbiological assay techniques (1). However, the difference between normal and myelogenous leukemic sera was not described by the above workers since they found the alpha-1 and alpha-2 globulins of normal serum to be the chief binding proteins for added vitamin B_{12}. The vitamin B_{12} content of myelogenous leukemic serum is also bound to the alpha globulins (1). It is of interest that, following treatment, the alpha globulins containing the vitamin B_{12} content of the serum were cleared slowly from the circulation, whereas the alpha globulins responsible for the increased binding of added vitamin B_{12} disappeared rapidly from the serum. Patients with an increased UBBC due to diseases other than chronic myelogenous leukemia always had increased binding of vitamin

B_{12} by the alpha-2 globulins but only occasionally had elevated alpha-1 globulin binding. This would suggest that the increase in alpha-1 globulin binding of vitamin B_{12} is the more specific change in chronic myelogenous leukemia. The percentage Co^{60} vitamin B_{12} bound by the other three protein fractions of myelogenous leukemic sera was decreased, whereas the Co^{60} vitamin B_{12} bound in mμg. per ml. was slightly increased as compared with the increase in alpha-1 or alpha-2 globulin binding.

The cause of the increased concentration of vitamin B_{12} in chronic myelogenous leukemia serum is unknown. The rise in serum vitamin B_{12} concentration to levels greater than the TBBC of normal serum and the invariable increase in UBBC both suggest an etiological relationship between the increased binding capacity and the hypervitaminemia. If vitamin B_{12} is bound more firmly than normal by the binding substances in myelogenous leukemic sera, a decreased serum turnover of the vitamin with a resultant rise in its serum concentration would ensue. Rates of serum turnover of bound vitamin B_{12} have been determined in chronic myelogenous leukemia following the intravenous administration of Co^{60} vitamin B_{12} (15). However, the estimation of turnover rates for normal subjects was not possible due to the small amounts of bound Co^{60} vitamin B_{12} remaining in the serum after 24 hours. The absence of hypervitaminemia despite the increase in UBBC in diseases other than chronic myelogenous leukemia is somewhat against such a hypothesis. Hypervitaminemia could also result from an excess amount of vitamin B_{12} entering the serum without any change in the serum turnover rate of the vitamin. Myelogenous leukemic leukocytes dying intravascularly could be the source of the added vitamin (2, 16). Against such a hypothesis is the absence of an increased concentration of vitamin B_{12} in myelogenous leukemic leukocytes (2, 16). If the survival time of chronic myelogenous leukemic leukocytes was shorter than that found in the other leukemias, an increased amount of vitamin B_{12} could also be presented to the serum. However, studies of white cell survival have demonstrated that the leukocytes in chronic myelogenous leukemia have a longer survival time than those of the acute leukemias (17).

The chemical nature of the vitamin B_{12} binding substances in myelogenous leukemic sera moving electrophoretically with the alpha globulins is also obscure. Whether these substances are proteins found normally in serum, but increased in chronic myelogenous leukemia, proteins of the serum that have been altered by the disease process, or abnormal tissue proteins foreign to normal serum, cannot be answered by this study.

SUMMARY

1. An equilibrium dialysis method for the determination of the vitamin B_{12} binding capacity of serum (UBBC) has been described. The serum concentration of vitamin B_{12}, the UBBC, and the total vitamin B_{12} binding capacity (TBBC) of sera from normal subjects and patients with chronic myelogenous leukemia, other leukemias and leukocytoses have been determined.

2. In normal subjects, the average serum vitamin B_{12} concentration was equal to 0.64 mμg. per ml., the UBBC to 1.51 mμg. per ml., and the TBBC to 2.23 mμg. per ml.

3. Patients with chronic myelogenous leukemia had an average serum vitamin B_{12} concentration of 8.09 mμg. per ml., a UBBC of 8.87 mμg. per ml., and a TBBC of 16.86 mμg. per ml. Following treatment, the UBBC fell rapidly to normal, while the decline in serum vitamin B_{12} concentration was a delayed and more gradual one.

4. Two patients with chronic monocytic leukemia, and one patient with leukocytosis had an increase in serum vitamin B_{12} concentration and UBBC. Six of eight patients with leukocytosis and one of three patients with acute leukemia had normal serum vitamin B_{12} levels but elevated UBBC's. Patients with chronic lymphocytic leukemia had a concentration of vitamin B_{12} and UBBC within the normal range.

5. The beta globulins bound an average of 44 per cent of the added Co^{60} vitamin B_{12} (average, 0.79 mμg. per ml.) and was the chief binding protein in 16 of 18 normal subjects. The alpha-2 globulin was next in vitamin B_{12} binding ability (an average of 28 per cent of the added Co^{60} vitamin B_{12} and 0.59 mμg. per ml.). The alpha-1 globulins bound the least Co^{60} vitamin B_{12} of any of the five protein fractions.

6. The alpha-1 globulins were the chief binding protein in 5 of 10 patients with chronic myeloge-

nous leukemia, binding an average of 38 per cent of the added Co^{60} vitamin B_{12} (3.55 mμg. per ml.). The alpha-2 globulins were the chief binding protein in 4 of the 10 patients, binding an average of 34 per cent of the added Co^{60} vitamin B_{12} (3.07 mμg. per ml.). With treatment of the disease, the pattern of vitamin B_{12} binding by the serum proteins returned towards normal.

7. An increase in the binding of Co^{60} vitamin B_{12} by the alpha-1 and alpha-2 globulins was noted in one patient with chronic monocytic leukemia, and one other patient with leukocytosis. Four other patients with elevated UBBC's of varying degree, due to diseases other than chronic myelogenous leukemia, had an increase in vitamin B_{12} binding by the alpha-2 globulins but no increase in alpha-1 globulin binding.

8. In addition to an increased serum concentration of, and binding capacity for, vitamin B_{12} in chronic myelogenous leukemia, there is an abnormal distribution of added vitamin B_{12} among the serum protein fractions in this disease.

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A COMPARATIVE STUDY OF INTRAPULMONARY GAS MIXING AND FUNCTIONAL RESIDUAL CAPACITY IN PULMONARY EMPHYSEMA, USING HELIUM AND NITROGEN AS THE TEST GASES [1, 2]

By JOHN B. HICKAM AND REGINA FRAYSER

(From the Department of Medicine, Duke University School of Medicine, Durham, N. C.)

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In describing the open circuit nitrogen method for estimating residual lung capacity, Darling, Cournand, and Richards (1) pointed out that imperfect gas mixing in the lung could impair the accuracy of the method. More recent studies have demonstrated that intrapulmonary gas mixing can be markedly uneven in patients with pulmonary emphysema (2–7). The mixing defect in advanced emphysema is often severe enough to make it likely that the functional residual capacity could be underestimated significantly by the Darling method, using the customary seven minute washout period.

In this laboratory an open-circuit helium method has been used which was designed to be particularly suited to the measurement of functional residual capacity in persons with defective intrapulmonary gas mixing (6). The method also provides a measure of intrapulmonary gas mixing in the form of an estimate of the size and turnover rate of the most slowly ventilated portion of the lung. In some patients with advanced emphysema and impaired mixing, the method has yielded values for functional residual capacity and total lung volume which are unusually large by conventional standards (7). In order to clarify the extent to which impaired gas mixing can introduce error into the measurement of functional residual capacity by conventional means, a comparative study has been made of the helium and the Darling methods in normal and emphysematous subjects. In order to evaluate the extent to which the measurement of functional residual capacity and intrapulmonary gas mixing by the helium method might be affected by the characteristics of the test gas, a study was also made of the effect of substituting nitrogen for helium as the test gas in this procedure. The present report describes the results of these studies. In general, the results confirm the suspicion that the Darling method can significantly underestimate the functional residual capacity in advanced emphysema and indicate that the values obtained by the helium method are not dependent upon special properties of the test gas.

METHODS

The method which employs helium for measuring the functional residual capacity (FRC) and gas mixing within the slowly ventilated portions of the lung has been described in detail elsewhere (6). The subject first breathes for 15 minutes a mixture of 50 per cent helium and 50 per cent oxygen. Then, at the end of a quiet expiration, he is switched to tank oxygen, and the expired gas is collected over the next seven minutes or more for subsequent measurement of its volume and helium concentration. The helium concentration of expired gas is followed continuously by means of a sensitive katharometer between levels of 3.00 and 0.05 per cent helium. When the concentration of expired helium is plotted against time on semilogarithmic paper, the resultant curve tends to form a straight line in the final part of its course. The appearance of this straight line indicates that those portions of the lung which are still excreting helium are being ventilated at similar enough rates to behave as though they constituted, in the aggregate, a single homogeneously ventilated space, which, for convenience, has been called the "slow space." From the slope and position of the terminal straight line and from the total minute ventilation volume (Vt) of the subject it is possible to calculate values for the volume (Vs) and ventilation rate (Vs) of the slow space. It is also possible to estimate the amount of helium remaining in the lung at any time after the slope and position of the straight-line part of the concentration-time curve has become clearly defined. For measurement of the FRC, the amount of helium contained within the lung at the start of the washout procedure is determined as the sum of that contained in the collecting bag and that estimated by graphic means still to be contained within the lung at the time collection is stopped. Where intra-

[1] This investigation was supported by a research grant, H-1370(C3), from the National Heart Institute, Public Health Service.

[2] This paper was presented at the Forty-seventh Annual Meeting of the American Society for Clinical Investigation, Atlantic City, N. J., May 2, 1955.

pulmonary gas mixing is very slow, a correction can be made for failure of the slow space to equilibrate completely with the inhaled helium mixture during the preliminary 15 minute period. When this correction is made, it increases the value obtained for the FRC. In the present study the largest value for this correction was 400 ml. (Case 7, Table II), and the average value of the correction for Cases 1 to 13 (Table II) was 120 ml.

When nitrogen was used as the test gas during the present study, a similar open-circuit arrangement was employed. The initial concentration of nitrogen in the lungs was assumed to be 81 per cent, and tank oxygen was used as the washing gas. The nitrogen concentration of mixed, expired gas was followed continuously by a nitrogen meter [3] between levels of 5.0 and 0.2 per cent N_2. As with the helium analysis, the sampled gas was drawn through an Ascarite® train on the way to the nitrogen meter for removal of water vapor and CO_2. The volume of this train was adjusted to provide a 25 second delay between sampling point and meter, to correspond to the time lag in the helium analysis. During the nitrogen washout procedure, the meter was read directly at appropriate intervals, or its output was amplified and recorded by a direct writer. Used as described, over this narrow range, the nitrogen meter proved to be quite reliable and stable. Calibration of the instrument was carried out with gas samples prepared by mixing known volumes of nitrogen and tank oxygen in a water displacement apparatus. With the nitrogen meter set as though tank oxygen contained no nitrogen, a linear relationship was found between the calculated nitrogen concentrations of the mixtures and the values as read from the nitrogen meter for the range 0.1 to 5.5 per cent N_2 (N_2 per cent equals meter reading plus 0.02). Over this range the standard deviation from regression was 0.04 per cent nitrogen. The same standard deviation from regression was found for the measurement of helium concentration by the katharometer in the range 3.00 to 0.05 per cent helium. To correct for the effect of absorbing approximately 2.5 per cent CO_2 by the Ascarite® train before analysis of expired gas, the readings of both helium and nitrogen were multiplied by the factor of 0.975.

For the measurement of FRC by the open-circuit method of Darling, which was carried out as a separate procedure, the expired gas was collected during seven minutes of oxygen breathing for subsequent determination of its volume and nitrogen concentration. Nitrogen concentration of the gas in the bag was measured by the nitrogen meter, using the Ascarite® absorber, as described above. During the washout, the intake valve of the nitrogen meter was connected into the mouthpiece so that the subject's expired air could be directly sampled without delay, drying, or CO_2 absorption. This arrangement allowed recording of the highest nitrogen concentration achieved during a prolonged, forced expiration at the end of the seven minute washout period. This value was used as the alveolar nitrogen concentration in calculating the FRC by the Darling method. For this analysis the nitrogen meter was "set" with gas mixtures of known nitrogen concentration which were saturated with water vapor rather than dry. It was necessary to use a different calibration line because of the presence of carbon dioxide and water vapor in the alveolar sample. This calibration line was derived from the analysis of 15 alveolar gas samples delivered by normal subjects after short periods of breathing oxygen. As determined on the Scholander apparatus, the nitrogen concentration of these samples ranged between 1.0 and 12.3 per cent and the CO_2 was in the vicinity of 5 to 6 per cent. Calculation of the regression line yielded the expression:

$$N_2 \text{ per cent equals } \frac{\text{meter reading plus } 0.24}{0.96},$$

with a standard deviation from regression of 0.19 per cent. To estimate the sensitivity of this calibration to changes in CO_2 concentration, a regression line was also calculated for a series of samples made by adding nitrogen to a gas mixture of approximately 10 per cent CO_2, 90 per cent O_2, with a subsequent Scholander analysis (8). For nitrogen concentrations between 2.0 and 17.0, the result was:

$$N_2 \text{ per cent equals } \frac{\text{meter reading plus } 0.65}{0.96},$$

with a standard deviation of 0.12 per cent. It is apparent that use of the calibration curve obtained with normal subjects might slightly underestimate the alveolar nitrogen concentration of a subject with hypercapnia, but the error introduced into the Darling method would not be great.

From the data of Behnke and Willmon (9), and of Jones (10), corrections were made for the amount of helium and nitrogen contributed by the body tissues during the washout period. This was done by constructing curves for the rate of elimination of test gases from the body tissues as a function of the duration of washout, taking into account the short equilibration period with helium. This correction is necessarily crude. To indicate the magnitude of the correction, the cumulative tissue contribution after 7, 15, and 30 minutes of washout was estimated to be, respectively, for nitrogen 220, 320, and 430 ml., and for helium 40, 60, and 80 ml. The usual corrections were made for apparatus dead space.

Calculations have been described elsewhere (6).

Observations were made on 21 male patients with obstructive pulmonary emphysema. The diagnosis was based on clinical and roentgenologic findings. In most cases, but not in all, the disease was far advanced. In 13 of the patients the FRC was measured both by the Darling method, using nitrogen, and by the prolonged washout method, using both helium and nitrogen. For the most part these measurements were all made at a single session, but in a few cases two sessions were needed. Rest periods of not less than 30 minutes were interposed between determinations by the different methods. The usual order of procedure was: seven minute

[3] Waters-Conley.

nitrogen, prolonged nitrogen washout, and prolonged helium washout. All measurements were made with the subjects in a sitting position. In 11 normal male volunteers, the FRC was measured both by the Darling method and the helium washout method.

RESULTS

The results obtained with the normal subjects are summarized in Table I. The mean lung volumes are similar to those reported elsewhere for normal subjects (11). In particular, there is good agreement between the FRC values measured by the Darling method and those measured by the helium washout method.

Table II summarizes the data obtained from the patients with emphysema. In general, these subjects had advanced emphysema as suggested by the large ratios of residual lung volume to total capacity. There is good agreement between the mean FRC measurements by the helium and the nitrogen washout methods. However, the mean FRC by the seven minute washout and alveolar N_2 method (Darling) was 0.53 liters less than the mean value yielded by the helium washout method. The difference is significant ($p < 0.01$). In five cases the volume by the helium method was more than 0.8 liters greater than that by the Darling method. Figure 1 relates the difference between these two measurements to the severity of the emphysema as indicated by the ratio of residual lung volume to total lung capacity (helium estimate). There appears to be a general tendency for the difference to increase as the severity of the emphysema increases.

There was no consistent difference between the size and ventilation rate of the slow spaces in emphysematous subjects as measured by helium washout on the one hand, and nitrogen washout on the other. Since the speeds of diffusion of gases are inversely proportional to the square roots of their densities (Graham's law), helium would be expected to "wash out" of a slow space more rapidly than nitrogen if the rate of gas diffusion were a limiting factor in the ventilation of such spaces. The expectation that helium and nitrogen would give different results for slow space characteristics under certain circumstances was investigated with a model lung and slow space. As shown in Figure 2, the "lung" consisted of a box which was ventilated by gas entering in a

TABLE I

Comparison of functional residual capacity measurements by the Darling and the helium washout methods—Normal subjects

Subject	Age	Surface area (M^2)	Alveolar N_2% after 7 min. O_2	FRC* by 7 min. washout and alveolar N_2 ($L.$)	FRC* by helium washout ($L.$)	FRC* by He less FRC by N_2 ($L.$)	$\dot{V}_E$ (min. ventilation during washout ($L./min.$)		Residual lung volume ($L.$)		Total lung capacity ($L.$)		RLV/TLC (%)	
							He	N_2	He	N_2	He	N_2	He	N
1. R. V.	22	1.73	0.7	3.36	3.48	0.12	9.1	10.6	1.50	1.38	6.42	6.30	23	22
2. H. C.	29	1.87	1.2	3.16	2.58	−0.58	11.4	11.6	1.42	2.00	6.05	6.63	24	30
3. B. F.	26	1.76	1.0	2.95	3.39	0.44	9.3	10.3	1.19	0.75	6.21	5.77	19	13
4. A. W.	21	2.04	0.7	2.92	2.92	0.00	8.7	9.1	1.01	1.01	6.34	6.34	16	16
5. S. M.	24	1.89	0.8	3.43	3.34	−0.09	10.8	10.0	1.37	1.46	5.81	5.90	24	25
6. T. H.	25	1.92	0.6	3.53	3.20	−0.33	9.3	10.0	1.13	1.46	6.25	6.58	18	22
7. P. K.	26	1.81	0.9	3.97	3.76	−0.21	9.6	8.1	1.42	1.63	6.97	7.18	20	23
8. D. W.	24	1.98	0.7	3.37	3.26	−0.11	10.7	10.6	0.97	1.08	6.79	6.90	14	16
9. B. C.	29	1.84	0.6	3.36	3.43	0.07	11.4	10.4	1.61	1.54	6.43	6.36	25	24
10. K. M.	23	2.12	0.5	3.17	3.30	0.13	10.1	10.8	1.52	1.39	6.60	6.47	23	22
11. B. S.	22	1.77	0.4	3.53	3.30	−0.23	11.1	9.8	1.71	1.94	6.14	6.37	28	30
Mean			0.7	3.34	3.27	−0.07	10.1	10.1	1.35	1.42	6.36	6.44	21	22
S. D.			0.2	0.31	0.32	0.27	1.0	0.9	0.24	0.37	0.33	0.40	4	5

* FRC = Functional residual capacity.

TABLE II

Measurement of the functional residual capacity by Darling and washout methods and comparison of "slow space" characteristics as measured by helium and by nitrogen—Emphysematous patients

Subject	Age	Surface area (M^2)	Alveolar N_2 % after 7 min. O_2	FRC* by 7 min. wash and alv. N_2 (*L.*)	FRC* by prolonged washout (*L.*)		FRC* by He less FRC by 7 min. N_2 (*L.*)	Vs ("Vol. of "slow space") (*L.*)		f (Vs/FRC)		k (Vs/Vs)		$\dot{V}t$ (min. ventilation during washout) (*L./min.*)		Residual lung volume† (*L.*)		Total lung capacity (*L.*)		RLV/TLC (%)		99% washout time‡ (*min.*)	
					He	N_2		He	N_2	He	N_2	He	N_2	He	N_2	He	N_2	He	N_2	He	N_2	He	N_2
1. E. A.	64	1.51	1.3	5.46	5.53	5.78	0.07	2.12	1.75	0.38	0.30	0.22	0.14	13.2	13.2	4.33	4.58	6.89	7.14	63	64	5.2	4.9
2. J. B.	61	2.00	2.2	4.26	4.61	4.52	0.35	3.30	2.14	0.71	0.47	0.55	0.43	14.5	15.5	3.08	2.99	7.32	7.25	42	41	4.2	4.1
3. D. O.	56	1.81	3.9	4.46	4.06	4.06	−0.40	2.37	2.38	0.58	0.59	0.27	0.28	12.0	13.0	3.52	3.52	5.57	5.57	63	63	5.7	5.9
4. W. L.	71	1.45	6.1	4.20	4.49	4.02	0.29	3.72	2.94	0.83	0.73	0.25	0.22	13.0	13.9							7.8	7.2
5. K. S.	67	1.94	3.2	5.29	6.13	5.94	0.84	5.32	2.76	0.87	0.46	0.32	0.21	11.3	12.5	5.01	4.82	7.77	7.58	65	64	7.9	7.8
6. C. W.	61	2.09	7.4	5.16	5.26	5.03	0.10	2.42	4.16	0.45	0.83	0.23	0.47	15.7	15.6	3.62	3.44	7.23	7.05	50	49	4.9	5.4
7. E. N.	36	1.61	5.6	4.29	5.78	5.71	1.49	4.00	4.35	0.69	0.76	0.16	0.16	8.1	10.4	5.22	5.15	7.32	7.25	71	71	11.8	12.0
8. S. Y.	64	1.88	3.2	5.31	5.76	6.15	0.45	2.08	4.09	0.36	0.66	0.25	0.25	12.4	13.0	5.14	5.53	7.11	7.50	72	74	5.2	7.4
9. G. F.	63	1.98	6.7	4.62	5.72	5.73	1.10	4.39	5.17	0.76	0.90	0.15	0.17	12.0	11.7	5.14	5.15	6.70	6.71	77	77	9.9	12.7
10. R. D.	59	1.80	3.5	5.44	6.38	6.40	0.94	4.12	5.56	0.64	0.87	0.24	0.26	13.5	14.7	4.89	4.91	8.23	8.25	59	60	7.8	8.7
11. D. W.	60	1.35	5.8	4.29	5.36	5.18	1.07	4.39	4.62	0.81	0.89	0.18	0.22	11.6	12.4	4.99	4.81	6.15	5.97	81	81	10.0	10.9
12. M. F.	51	1.69	5.2	5.20	5.30	5.59	0.10	3.94	3.98	0.74	0.71	0.53	0.46	15.1	15.0	3.97	4.26	7.57	7.86	52	54	4.4	5.4
13. F. H.	67	1.83	4.7	4.28	4.78	4.93	0.50	2.26	4.00	0.47	0.81	0.33	0.37	15.2	14.3	4.23	4.38	6.02	6.17	70	71	4.2	6.2
Mean			4.5	4.79	5.32	5.31	0.53	3.42	3.68	0.64	0.69	0.28	0.28	12.9	13.5	4.43	4.46	6.99	7.03	64	64	6.8	7.6
nos. 1–13			±1.8§	±0.52	±0.68	±0.77	±0.53	±1.06	±1.18	±0.17	±0.19	±0.13	±0.12	±2.0	±1.5	±0.74	±0.78	±0.77	±0.79	±12	±12	±2.5	±2.8
14. G. F.‖	63	1.98			5.23	5.21		4.01	3.64	0.77	0.70	0.14	0.16	11.9	12.1	4.55	4.53	6.63	6.61	69	68	9.3	10.7
15. C. W. T.	67	1.86			5.66	5.41		3.22	3.72	0.57	0.69	0.27	0.27	12.9	11.0	4.83	4.68	7.37	7.22	66	65	6.6	8.6
16. J. R.	69	1.75			4.63	3.87		1.60	2.00	0.35	0.59	0.09	0.30	14.0	14.1	4.51	3.75	5.85	5.09	77	74	5.5	5.0
17. L. A.	58	1.73			4.36	4.51		2.20	1.57	0.50	0.35	0.49	0.20	11.4	11.5	2.91	3.06	5.92	6.07	49	50	4.6	5.9
18. F. S.	64	1.66			6.64	6.41		4.71	4.82	0.71	0.75	0.28	0.22	18.3	11.5	6.10	5.87	7.99	7.76	76	76	6.8	10.0
19. A. K.	74	1.84			4.64	4.68		2.53	2.44	0.55	0.52	0.32	0.14	12.3	12.4	4.22	4.26	6.46	6.42	65	66	6.0	7.2
20. J. L.	61	1.94			5.06	4.39		3.97	2.94	0.77	0.67	0.28	0.35	10.7	11.2	4.44	3.77	6.64	5.47	67	69	8.3	6.0
21. T. B.	75	1.67			4.48	5.14		2.72	3.04	0.61	0.59	0.33	0.17	14.6	13.3	3.28	3.94	6.30	6.96	52	57	5.5	8.3
22. E. A.	59	1.74			3.95	4.48		1.80	3.00	0.46	0.67	0.35	0.27	11.5	11.8	2.81	3.34	5.41	5.98	52	57	4.8	7.2
Mean					5.17	5.14		3.24	3.41	0.62	0.66	0.28	0.26	13.0	12.9	4.32	4.32	6.78	6.76	64	64	6.7	7.6
nos. 1–22					±0.74	±0.77		±1.07	±1.13	±0.16	±0.16	±0.12	±0.10	±2.1	±1.5	±0.87	±0.80	±0.80	±0.86	±11	±10	±2.2	±2.4

* FRC = Functional residual capacity.

† Based on FRC values obtained by prolonged washout.

‡ The time required for the concentration of test gas in the mixed, expired air to fall to 1 per cent of the original concentration in the lung. Correction has been made for estimated tissue excretion but not for failure to "fill" completely with helium prior to washout.

§ Ranges below means are standard deviation.

‖ Study No. 9 on G. F. precedes Study No. 14 on the same subject by two months.

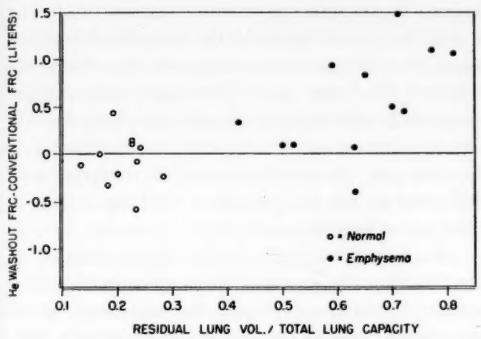

FIG. 1. DIFFERENCE BETWEEN HELIUM WASHOUT AND CONVENTIONAL OPEN-CIRCUIT FUNCTIONAL RESIDUAL CAPACITY MEASUREMENTS IN NORMAL AND EMPHYSEMATOUS SUBJECTS

In the normal subjects there is good overall agreement between the two methods, but in the subjects with emphysema the results by the helium method are, as a group, significantly greater than those by the method of Darling, Cournand, and Richards.

steady flow through holes in corner pipes and leaving by a central exhaust pipe. The two different "slow spaces" consisted of hollow inserts placed within the lung. The "convection" insert communicated with the surrounding lung by numerous holes 1 mm. in diameter. Ventilation of the surrounding lung produced slow convection currents in the insert, made visible by filling it with smoke. The "diffusion" insert was a cylinder closed at the ends by two Grade IV sintered bronze "porex" discs described by the manufacturer [4] as having pores with a general size range of 0.0001 to 0.0005 inch in diameter. Ventilation of the surrounding lung produced no visible convection currents in the insert. Ventilation of this insert must have involved to some extent the process of gas diffusion through the pores. Using nonventilated dummy inserts, flow through the lung was first adjusted to provide substantially uniform ventilation in the space around the insert as judged by the washout curves. Oxygen was used as the washing gas. For helium washouts, the lung and insert were filled at the start with 50 per cent helium and 50 per cent oxygen, and for nitrogen washouts they were filled with air. The results obtained with the two different inserts and the two different test gases are presented in Table III. The slow space caused by the "convection" insert had substantially the same size and turnover rate when measured by helium as when measured by nitrogen. However, with the slow space caused by the "diffusion" insert, the turnover rate by helium was considerably faster than by nitrogen. This result confirmed the expectation that the two different test gases might give different slow space characteristics in cases where the process of diffusion was a limiting factor in slow space ventilation.

[4] Moraine Products Division, General Motors Corporation.

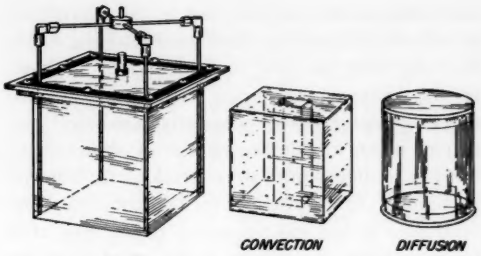

FIG. 2. MODEL LUNG WITH "SLOW SPACE" INSERTS
For description, see text.

DISCUSSION

The essential difference between the helium washout and Darling methods for measuring

TABLE III

Influence of the nature of the test gas on apparent characteristics of the slow space in a model lung

Slow space insert	Test gas	Vt Volume of lung and insert (*ml.*)	Volume of insert (*ml.*)	$\dot{V}t$ Total ventilation rate (*ml./min.*)	Vs Calculated slow space volume (*ml.*)	$\frac{k}{\dot{V}s/Vs}$	$\frac{f}{Vs/FRC}$
"Convection"	Helium	1,980	910	2,400	830	0.26	0.42
	Nitrogen	1,980	910	2,400	920	0.22	0.46
"Diffusion"	Helium	2,080	830	2,400	1,090	0.77	0.52
	Nitrogen	2,080	830	2,400	1,280	0.46	0.61

functional residual capacity lies in the means used to estimate the amount of test gas remaining within the lung after the first few minutes of washout. In the helium method the expired air is continuously monitored by a sensitive analytical system to insure that measurement of the excreted test gas continues until the excretion rate becomes negligible. In the Darling method the mean concentration of test gas remaining in the lung after seven minutes of washing is estimated from the composition of an alveolar air sample. The present results indicate that in patients with the severe mixing defect of advanced emphysema, the alveolar air method significantly underestimates the amount of test gas remaining within the lung and consequently underestimates the functional residual capacity, sometimes by a considerable quantity. It is apparent from these and from previous results (6, 7) that the poorly ventilated lung regions in such patients usually make a relatively small contribution to alveolar air samples. The contribution is often large enough in patients with advanced emphysema to yield an abnormally high nitrogen concentration in the seven minute alveolar sample of the Darling method, as found by Cournand, Baldwin, Darling, and Richards (12). There is, however, no consistent relationship in the present patients between the height of the seven minute alveolar nitrogen concentration and the severity of the mixing defect, as judged by the amount of nitrogen remaining in the lungs in excess of that estimated from the alveolar sample (Table II).

Using a plethysmographic method for the measurement of thoracic gas volume, Bedell, Marshall, DuBois, and Comroe (13) have found values in patients with emphysema sometimes considerably in excess of the functional residual capacity yielded by the Darling method, while the agreement was quite good in patients with some other pulmonary disorders and in normal subjects. Since this method can measure noncommunicating gas volumes, such as closed cysts, it would also be expected to give results larger than those by the helium method in some cases of emphysema. These results and others indicate that the functional residual capacity and total lung volume in severe emphysema are generally larger than previously thought.

The finding that nitrogen can be substituted for helium in the washout method without producing a significant difference in the functional residual capacity or a consistent change in the characteristics of the "slow space" in emphysematous patients indicates that the results with this method are not dependent upon special characteristics of the test gas. It also indicates that the process of diffusion is not in general a limiting factor in slow space ventilation.

The results with the model draw attention to the fact that the apparent characteristics of a slow space depend in part upon the mechanics of its ventilation. It was pointed out by Robertson, Siri, and Jones (2) that if a slow space is ventilated in parallel with the rest of the lung, its size and turnover rate can be well estimated by following the rate at which it excretes a test gas, as in the present method. However, if it is ventilated in series, in the sense that it exchanges gas with a faster ventilated space which in turn exchanges with the outside, it is imperfectly characterized by the present method. Series ventilation makes a slow space appear bigger and slower than it actually is, but the apparent size is affected more than the turnover rate. The distortion is small when the slow space is ventilated much more slowly than the fast space with which it is exchanging, but as the turnover rates become similar, distortion becomes large. This effect may account in part for the tendency of the method to overestimate the size of the diffusion insert, which has a relatively fast turnover rate (Table III). Series ventilation must certainly occur to some extent in both normal and diseased lungs. In the usual patient with emphysema, the representation of the slow space is probably fairly accurate because of the great difference between its ventilation rate and that of the rest of the lung. In normal subjects, however, slow spaces are much more rapidly ventilated and consequently they will be somewhat overemphasized by the occurrence of series ventilation.

SUMMARY

1. A comparative study has been made of the measurement of functional residual capacity by the method of Darling, Cournand, and Richards and by the open circuit helium washout method in normal subjects and in patients with pulmo-

nary emphysema. In the normal subjects, agreement between the two methods was good, but in the patients with emphysema a significantly larger mean value was obtained by the helium method than by the Darling method. This discrepancy appears to result from the inability of an alveolar air sample to represent adequately the mean intrapulmonary gas concentration in patients with severe defects in intrapulmonary gas mixing.

2. Substitution of nitrogen for helium in the washout procedure made no significant difference in the measurement of functional residual capacity and produced no consistent changes in the apparent characteristics of the slowly ventilated lung spaces of patients with emphysema.

3. The rate of gas diffusion is apparently not a limiting factor in the ventilation of the large, slowly ventilated lung regions of patients with pulmonary emphysema.

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TISSUE DISTRIBUTION OF C^{14} AFTER THE INTRAVENOUS INJECTION OF LABELED CHYLOMICRONS AND UNESTERIFIED FATTY ACIDS IN THE RAT

By JOSEPH H. BRAGDON and ROBERT S. GORDON, Jr.

(From the Laboratory of Cellular Physiology and Metabolism, National Heart Institute, National Institutes of Health, Bethesda, Md.)

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Chylomicrons, labeled in the triglyceride moiety with palmitic acid-1-C^{14}, were injected intravenously into fasting dogs by Havel and Fredrickson (1). They showed that the chylomicrons were rapidly removed from the plasma. The radioactivity did not reappear to any significant degree in the alpha or beta lipoproteins of the plasma, but did reappear as unesterified fatty acid bound to albumin (UFA). There was no actual increase in UFA concentration but the specific activity of this fraction exceeded 10 per cent of that of the the triglyceride fatty acid injected. They concluded that hydrolysis of the chylomicrons occurred in some extravascular compartment. It therefore seemed desirable to determine if possible the anatomic sites of removal of the chylomicrons. To this end, rats were injected with rat chylomicrons labeled in the triglyceride moiety with palmitic acid-1-C^{14}, and the tissue distribution of the label was compared with its distribution following the injection of palmitic acid-1-C^{14} bound to albumin (UFA).

We have previously reported (2) that although the nutritional state of the rat does not influence the rate of removal of chylomicrons from the blood, carbohydrate feeding strongly inhibits the oxidation of both chylomicrons and albumin-bound UFA (3, 4). We therefore decided to determine whether carbohydrate feeding would influence the tissue distribution of the injected chylomicrons or UFA.

METHODS

Donor rats, the cysternae chyli in which had been cannulated on the previous day, were given about 50 μc. of potassium palmitate-1-C^{14} in a few ml. of water by stomach tube. The donor rats were offered their usual commercial pellet diet and physiologic saline to drink. No carrier fat was added to the diet of the donor for Experiment I, but olive oil was mixed with the diet of the donor for Experiment II. The chyle was collected in an ice bath. The following day, after expression of the clot, the chyle was layered under 0.15 M salt solution and centrifuged for 30 minutes at approximately 100,000 times G. The chylomicrons were thereby concentrated at the top of the centrifuge tube, where they were readily separated from the residual chyle proteins. They were washed once more through saline by a second centrifugation. Analysis of one preparation showed that 97 per cent of the radioactivity was contained in the triglyceride moiety, and that most of the remainder was in the phospholipids, thus confirming the findings of previous investigators (1, 5).

UFA was prepared for injection as follows: Approximately 3 mg. of palmitic acid-1-C^{14} containing about 10 μc. of radioactivity was dissolved in 1 ml. of ethanol in a small flask. A moderate excess of 0.02 N NaOH was added, and the mixture evaporated to dryness. The residue of sodium palmitate was dissolved in 1 ml. of distilled water by gentle heating, cooled to approximately 40° C., and 9 ml. of rat serum at 4° C. was added suddenly with vigorous agitation. No crystallization of sodium palmitate was observed. The serum was submitted to paper electrophoresis in sodium barbital buffer at pH 8.6. The dried paper strip was scanned for radioactivity and then stained for protein. The distribution of radioactivity coincided with that of albumin.

In Experiment I, in which the recipient rats were to be killed only 10 minutes after injection, it was anticipated that significant quantities of the injected chylomicrons might still be present in the recipients' plasma. It therefore became desirable to know the quantity of plasma present in each tissue so that the radioactivity contributed by the plasma could be subtracted from the observed tissue activity to give the amount of activity actually cleared by that tissue. For that purpose, about 50 μc. of radioiodinated human serum albumin (RISA®) was added to 1 ml. of normal rat serum, the mixture incubated at room temperature, and exhaustively dialyzed to remove unbound I^{131}. This material was then added to 5 ml. of washed chylomicrons and to 5 ml. of the UFA preparation. In Experiment II, in which the recipient rats were to be permitted to survive 200 minutes after injection, no radioiodinated serum albumin was used.

The experimental animals were 12 male Sprague-Dawley rats, weighing between 210 and 225 grams at the time of injection. Food was removed from their cages 20 hours previously. Carbohydrate-fed animals were offered 10 per cent glucose in half-strength physiologic saline. During the night preceding the experiment, each consumed at least 120 ml. of this preparation. In Ex-

TABLE I

The specific activity of tissues 10 minutes after the injection of C^{14}-labeled chylomicrons or unesterified fatty acid*

Rat No.	1	2	3	4	5	6	7	8
Nutritional state	CHO†	CHO	CHO	CHO	Fast	Fast	Fast	Fast
Lipid injected	Chylo‡	Chylo	UFA§	UFA	Chylo	Chylo	UFA	UFA
Liver	12.0	10.2	24.3	17.9	19.0	23.4	12.7	17.1
Muscle	1.6	1.6	1.9	2.1	0.7	0.4	1.2	1.5
Fat	12.4	12.6	0.5	0.3	1.3	2.9	0.8	0.6
Spleen	8.2	6.9	4.0	4.3	7.2	14.0	1.7	1.8
Lung	4.3	5.9	10.2	14.1	3.9	5.1	5.6	6.8
Kidney	1.8	2.2	7.4	7.9	1.9	3.3	3.6	5.9
Heart	4.8	4.6	16.0	16.0	15.1	22.0	3.0	4.1
Brain	0.1	0.0	0.3	0.4	0.3	0.5	0.3	0.4

* Expressed as cpm times 10^{-3} per gram wet weight corrected for plasma content and for dose.
† CHO = Carbohydrate-fed.
‡ Chylo = C^{14}-labeled chylomicrons.
§ UFA = Unesterified fatty acid.

periment I, each received 2 ml. of 50 per cent glucose solution by stomach tube one hour before the injection. The fasted animals were offered half-strength saline and similarly received water by stomach tube.

Each animal received approximately 1 μc. of C^{14} as either chylomicrons or UFA in a total volume of 1 ml. by injection into a tail vein. The chylomicron dose expressed as total lipid (6) was 18 mg. in Experiment I and 44 mg. in Experiment II. After 10 minutes and 200 minutes, respectively, in the two experiments, the animals were anesthetized with intravenous sodium pentobarbital. Five ml. of blood was withdrawn from the abdominal aorta into a syringe containing dry heparin. The organs and tissues were removed, washed in saline, blotted dry, weighed, and cut up into ethanol-acetone (1:1) as rapidly as possible in the following order: piece of liver, spleen, epididymal fat bodies, kidneys, right adductor muscle, heart ventricles, lungs, and brain. The remainder of the liver was weighed and discarded. The carcass and remaining viscera were put through a meat grinder and then extracted in 3 liters of ethanol-acetone in a Waring blendor for three minutes. All tissues were in fixatives within 10 minutes following the injection of anesthetic. The organ and tissue samples were extracted in about 45 ml. of ethanol-acetone in a smaller blendor, and the extracts were transferred with washings to graduated centrifuge tubes, where they were brought to a final volume of 50 ml. Ethanol-acetone extracts of the injected material and of the plasma were also made. On the following day, the extraction mixtures were centrifuged, and aliquots of the ethanol-acetone extracts were evaporated under air at 57° C. In the case of serum extracts, a drop of olive oil was added, as it had been previously demonstrated that UFA may sublime under these conditions if there is not sufficient carrier fat. The dried lipids were then taken up in a final volume of 10 ml. of toluene and centrifuged briefly for clarification. For C^{14} counting 3 ml. of the toluene extracts was added to 10 ml. of a 0.4 per cent solution of diphenyloxazole in toluene and counted at $-10°$ C. in a Packard Tri-Carb scintillation counter. In order to correct for any possible contamination of the toluene by I^{131}, another 3 ml. aliquot was counted in a thallium-activated sodium iodide well-type scintillation counter. I^{131} was found in only insignificant quantities and no corrections were necessary.

For determination of the I^{131} content of the tissues, the protein precipitates of the original extracts were dissolved in 10 ml. of 25 per cent KOH in 50 per cent ethanol. After standing for several days at room temperature, they were brought to a final volume of 15 ml. with water. All material dissolved completely, except

TABLE II

The recovery and distribution of C^{14} 10 minutes after the injection of labeled chylomicrons or unesterified fatty acid

Rat No.	1	2	3	4	5	6	7	8
Nutritional state	CHO*	CHO	CHO	CHO	Fast	Fast	Fast	Fast
Lipid injected	Chylo*	Chylo	UFA*	UFA	Chylo	Chylo	UFA	UFA
Per cent of dose recovered as lipid-soluble C^{14}	93	85	86	87	70	72	41	54
Per cent of dose remaining in plasma	38	40	1	1	41	36	1	1
Per cent of cleared activity found in:								
Liver	19	15	30	26	21	21	14	16
Muscle	23	23	29	31	9.7	5.7	16	21
Fat	25	36	1.3	0.8	3.6	4.3	1.1	1.1
Spleen	1.1	0.9	0.4	0.6	1.0	1.3	0.2	0.3
Lung	0.8	0.8	1.7	2.1	0.6	0.7	0.6	0.8
Kidney	0.3	0.6	1.8	1.7	0.5	0.7	0.9	1.2
Heart	0.5	0.5	1.6	1.6	1.5	2.2	0.3	0.3

* See footnotes to Table I for identification of these abbreviations.

TABLE III

The specific activity of tissues 200 minutes after the injection of C^{14}-labeled chylomicrons or unesterified fatty acid*

Rat No.	9	10	11	12
Nutritional state	CHO†	CHO	CHO	CHO
Lipid injected	Chylo†	Chylo	UFA†	UFA
Liver	11.0	11.4	6.4	6.6
Muscle	0.7	0.7	2.1	2.3
Fat	19.2	11.8	0.6	1.6
Spleen	2.9	3.7	2.7	4.5
Lung	3.0	3.1	6.6	8.4
Kidney	2.3	2.0	4.5	6.4
Heart	2.3	3.1	3.6	7.1
Brain	0.1	0.1	0.2	0.2

* Expressed as cpm times 10^{-3} per gram wet weight corrected for plasma content and for dose.
† See Table I for identification of these abbreviations.

the glycogen in the livers of carbohydrate-fed animals. Aliquots were counted in the well-type scintillation counter.

In some preliminary experiments, two portions of the same liver were compared. Similarly, perirenal fat was compared with epididymal fat in the same animal. The differences in C^{14} content per gram of tissue were of the same magnitude as those reported here between similarly treated animals.

RESULTS

The amount of plasma contained in each tissue sample was determined by the I^{131} content of the sample. The amount of C^{14} radioactivity contributed by this amount of plasma was then subtracted from the observed C^{14} activity. The C^{14} activity was also corrected for dose, so that the data for all animals are directly comparable. For this purpose, the "dose" was considered to be only that radioactivity which had been cleared from the blood. These corrections involved significant quantities only in the animals killed 10 minutes after receiving chylomicrons, as in all other animals the plasma contained relatively little radioactivity. The tissue specific activity, presented in Tables I and III, thus represents the observed C^{14} activity per gram of wet tissue corrected for plasma content and for "dose."

The recovery and distribution data are presented in Tables II and IV. The percentage of C^{14} recovered was determined from the sum of the observed radioactivity in the blood withdrawn, in each organ and tissue removed, and in the carcass. The percentage of the dose remaining in the plasma was calculated after multiplying the activity found in 1 ml. of plasma by the plasma volume, as determined by I^{131} dilution. The percentage of cleared activity found in the several tissues was derived by multiplying the activity per gram by the total weight of that tissue and dividing by the cleared "dose." Organ weights were determined directly. Body fat was determined by chemical analysis of the toluene carcass extract (6). No correction was made for water content of the sample of adipose tissue weighed. It should be realized that the fat extracted from the carcass included small amounts of fat from tissues other than adipose tissue. Muscle mass was estimated as 45 per cent of the body weight (7).

DISCUSSION

Recovery

The fact that 85 to 93 per cent of the injected activity were recovered after 10 minutes in the fed animals shows that the methods used were adequate. Ten minutes after injection in the fasting state, about 60 per cent of the chylomicron activity and 99 per cent of the UFA activity had been cleared from the blood. In both situations,

TABLE IV

The recovery and distribution of C^{14} 200 minutes after the injection of labeled chylomicrons or unesterified fatty acid

Rat No.	9	10	11	12
Nutritional state	CHO*	CHO	CHO	CHO
Lipid injected	Chylo*	Chylo	UFA*	UFA
Per cent of dose recovered as lipid-soluble C^{14}	72	66	51	63
Per cent of dose remaining in plasma	1	1	1	1
Per cent of cleared activity found in:				
Liver	14	17	6.8	9.8
Muscle	10	11	36	39
Fat	52	31	1.8	3.3
Spleen	0.3	0.5	0.2	0.5
Lung	0.4	0.4	1.0	1.2
Kidney	0.5	0.5	1.0	1.3
Heart	0.2	0.3	0.4	0.6

* See Table I for identification of these abbreviations.

about half of the cleared activity was no longer recoverable as lipid-soluble C^{14}. We have reported previously (3, 4) that in similarly treated rats approximately 5 and 19 per cent, respectively, of the injected radioactivity was expired as $C^{14}O_2$ within 10 minutes of injection. In the experiments reported here, more than 10 minutes elapsed from the time of injection until the processes of enzymatic degradation were halted in the tissues. It is very likely that much of the unrecovered activity was present in the form of bicarbonate and metabolic intermediates.

The sparing action of carbohydrate on fatty acid oxidation was first shown by Lossow and Chaikoff (8), who measured $C^{14}O_2$ excretion after the intravenous injection of palmitic acid-1-C^{14} both as free acid and as triglycerides in synthetic emulsions. In our previous reports (3, 4) we showed the marked sparing effect of carbohydrate feeding on the oxidation of palmitic acid when administered in the more physiologic forms of chylomicrons and UFA. Carbohydrate feeding in the present experiments permitted the recovery of over 50 per cent of the injected dose as lipid-soluble C^{14}, even after 200 minutes. The results of Experiment I confirm our previous report (2) that chylomicrons are cleared no more rapidly in the fasting animal than in the animal in positive caloric balance.

Mechanism of chylomicron clearance

In the dog experiments of Havel and Fredrickson (1) the radioactivity, the triglyceride content, and the phospholipid content of the chylomicron fraction of the recipients' sera decreased at the same rate, suggesting that the chylomicrons were being removed intact. Studies of the changes in concentration of certain low-density serum lipoproteins following alimentary hyperlipemia in human subjects have been interpreted, however, as indicating the conversion of chylomicrons into lipoproteins of increasing density (9). It was implied that lipid was being removed from the circulating lipoproteins. Although it has been suggested that the clearing factor is the enzyme responsible for this phenomenon, the enzyme can rarely be demonstrated in the plasma in appreciable quantities, except following the injection of heparin or heparinoids. In the present experiments, if intravascular hydrolysis of triglyceride to UFA were the major pathway for the clearance of chylomicrons, one would expect the distribution of labeled fatty acid to be the same whether injected as chylomicron or as UFA. Such was not the case. After chylomicron injection in carbohydrate-fed animals from 25 to 50 per cent of the cleared activity were found in the body fat, whereas after UFA injection, less than 4 per cent of the cleared activity appeared there, proving that chylomicrons were being removed by adipose tissue without prior hydrolysis.

Distribution of chylomicrons

In the carbohydrate-fed rats, even 200 minutes after the injection of chylomicrons, approximately 70 per cent of the injected radioactivity was recovered as lipid-soluble C^{14}. It is of interest to compare the tissue distribution of this activity at 200 minutes with that at 10 minutes. The specific activity of liver, adipose tissue, and kidney, corrected for activity actually cleared, remained essentially unchanged. That of muscle, spleen, lung, and heart decreased approximately 50 per cent. These findings indicate that during the time interval there had been relatively little redistribution of fatty acid activity. In the former group of tissues there had apparently been storage of chylomicron activity, and in the latter group there had been a loss of about 50 per cent of that cleared, probably by oxidation.

The distribution of chylomicrons in the fasting state can be compared with the distribution after carbohydrate feeding, only in the 10 minute experiment. In the fasting state, the specific activity in the liver and in the heart muscle are significantly higher than they are in the fed animals in spite of the fact that in the fasting state the total activity recovered was considerably less. These differences indicate that the nutritional state of the animal determines to some extent the sites at which chylomicrons are removed. The opposite finding, *i.e.*, less radioactivity in the muscle of the fasting animal than in that of the fed, does not carry the same implication, for it is possible that the amount cleared by muscle in the fasting animal was actually greater, but that more had been oxidized. In the case of fat, the specific activity in the fasting state was less than 20 per cent of the specific activity found in the fed animals. Again there are two possible explanations: either less was cleared by the depots in the fasting state,

or the same amount was cleared but the radioactivity was rapidly redistributed, presumably as UFA. Adipose tissue is known to be rich in the enzyme lipoprotein lipase (10). The caloric requirements of the depots are presumably so small that the possibility of local oxidation may be neglected. It is not necessary to assume that 80 per cent of the fatty acids of the depots underwent exchange in this short period of time, as the mixing of labeled fatty acid with depot fatty acid was probably not complete.

Distribution of unesterified fatty acid

The very rapid disappearance of 99 per cent of the labeled UFA from the circulating blood, and the long persistence of the remaining 1 per cent indicate that an exchange occurs between plasma UFA and the fatty acids of a tissue pool. This tissue pool must contain about 100 times as much exchangeable fatty acid as does the plasma compartment. Adipose tissue does not contribute significantly to this tissue pool, as is shown by the apparent size of the pool (adipose tissue contains about 10,000 times as much fatty acid as does the circulating UFA fraction) and by the failure of the injected labeled UFA to enter the depot fat, even in rats maintained for 200 minutes after injection. We suspect that the 10 minute distribution data are, in part, indicative of the relative blood flow of each tissue, whereas the 200 minute figures reflect more accurately the amount of exchangeable fatty acid present in the different tissues. Thus, at 10 minutes in carbohydrate-fed rats, the most heavily labeled tissues are liver, lung, kidney, and heart, whereas, at 200 minutes, the specific activity of muscle was comparable to those of the other tissues and because of its greater mass, muscle contained the largest fraction of the total recoverable activity. The tissue radioactivities observed in fasted animals at 10 minutes must represent a complex function of blood flow, tissue pool size, and oxidative rate, and cannot as yet be interpreted in detail.

SUMMARY

Rats were injected intravenously with either rat chylomicrons, labeled in the triglyceride moiety with palmitic acid-1-C^{14}, or with the unesterified labeled acid bound to serum albumin (UFA). Some of the recipient rats were fasting and some were carbohydrate-fed. They were killed 10 and 200 minutes after injection, and the tissues were analyzed for their C^{14} content. Carbohydrate feeding spared the oxidation of both lipid forms and greatly increased the recovery of lipid-soluble C^{14}. The tissue distribution of the chylomicron label was quite different from that of the UFA label. This was interpreted as proving that the bulk of the chylomicrons do not undergo intravascular hydrolysis, and as indicating that they are removed intact from the circulation. Cleared chylomicron activity in the fasting state was found primarily in the liver and, after carbohydrate feeding, primarily in the depots.

Plasma UFA rapidly exchanges with a tissue pool, which contains about 100 times as much exchangeable fatty acid as does the plasma. The fat depots are not a significant part of this pool, the bulk of which is in liver and muscle.

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ON THE MECHANISM OF OVERPRODUCTION OF URIC ACID IN PATIENTS WITH PRIMARY GOUT [1]

By JAMES B. WYNGAARDEN, ALBERTA E. BLAIR, AND LINNELLE HILLEY

(From the Department of Medicine, Duke University School of Medicine, Durham, N. C., and the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Md.)

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In order to study the rate of generation of uric acid in man, Benedict, Roche, Yü, Bien, Gutman, and Stetten (5–7) administered relatively large doses of glycine-N^{15} orally to control and gouty subjects and measured the appearance of isotope in urinary uric acid. With this technique, they demonstrated overincorporation of isotope into uric acid only in gouty subjects excreting large quantities of uric acid in urine. Subsequently, however, excessive incorporation of *tracer* quantities of glycine-1-C^{14} into uric acid was demonstrated in all of seven subjects studied, regardless of the stage of severity of the disease or of the magnitude of urinary uric acid excretion (8). These results indicated that overproduction of uric acid from glycine and other small molecules was the fundamental defect responsible for hyperuricemia in primary gout. The characteristics of the curves of uric acid enrichment indicated that excessive biosynthesis of uric acid occurred in these gouty subjects without the intermediary intervention of nucleic acids (5, 7, 8).

Since the mechanism of overproduction of uric acid in primary gout has not been defined in detail, it was decided to investigate the incorporation of glycine-1-C^{14} into various urinary purine bases and to compare enrichment patterns with those of uric acid determined simultaneously. From such comparisons, information was sought regarding purine intermediates involved in normal and abnormal uric acid production. These studies have suggested two types of mechanism resulting in uric acid synthesis in man and have strengthened the present concepts (5, 7, 8–12) that overproduction of uric acid may occur by one mechanism in primary gout and by another in proliferative hematopoietic disorders of the type associated with secondary gout.

[1] This work has been supported, in part, by grants from the National Institutes of Health, United States Public Health Service, Grant No. A-1391, and from the United Medical Research Foundation of North Carolina. Preliminary reports of this work have appeared in abstract form (1–3), and certain aspects have been included in a recent review article (4).

METHODS

Materials and instruments. Glycine-1-C^{14}, 1 to 2 mc. per mM, was purchased from Nuclear Instrument and Chemical Corporation, Chicago. Purified uricase, xanthine oxidase, and catalase were purchased from Worthington Biochemicals Corp. Guanase was prepared from rat liver according to Kalckar (13). Adsorption readings and enzymatic analyses were made in a Beckman DU ultraviolet spectrophotometer. Spectra were determined in a Beckman DK ultraviolet recording spectrophotometer. Chromatographic elution fractions were analyzed for compounds absorbing at 253 mμ in a Gilson ultraviolet scanner and traced by an Esterline-Angus recorder; fractions were collected in a Gilson volumetric fraction collector. C^{14} measurements were made in a Robinson gas-flow counter.

Subjects. Pertinent data on subjects of this study are given in Table I. All subjects ingested a purine poor diet for a preparatory period of about five days and for the duration of the study. This diet contained about 30 mg. of purine-N and 55 to 60 Gm. of protein. Glycine-1-C^{14} was administered in solution orally with breakfast to four control subjects, three subjects with primary gout, one subject with polycythemia vera and secondary gout, and one subject with myeloid metaplasia. Subjects received from 2.5 to 25 μc. of C^{14}.

Collections and analyses. Twenty-four hour urine collections were obtained under toluene at room temperature from the second day of dietary preparation and continuously thereafter for periods up to 24 days. When the urinary uric acid excretion had reached a stable low value, glycine-1-C^{14} was administered. Thereafter, samples were collected and stored at − 10° C. until analyzed. Uric acid analyses on urine and serum were performed by differential spectrophotometry (14, 15) employing purified uricase.

Urinary purines were precipitated with copper and dissolved in HCl according to the Hunter and Givens modification (16) of the procedure of Krüger and Schmid (17). On reduction of volume and chilling of the acid solution, uric acid precipitated and was col-

TABLE I

Data on subjects given glycine-1-C^{14}

Name Age, Sex, Race Wt. (*Kg.*)	Diagnosis	Dose *μc.*	Hemogram			Uric acid		Resumé
			Hgb.	Hct.	WBC	Serum *mg. %*	Urine *mg./day*	
C. L. 62, M, W 67.9	Osteoarthritis, rt. hip	5.0		45	9,500	4.1–4.4	355 ± 42	
H. B. 26, M, C 62.7	Contact dermatitis	25.0	14.0		8,700	3.8	231 ± 66	No stigmata or family history of gout or of hematopoietic disorders.
W. W. 21, M, W 67.3	Duodenal ulcer	13.6	15.2	42	4,850	4.4	346 ± 50	
E. S. 30, F, W	Psychoneurosis	5.0	13.8	41	4,950	5.1–5.7	236 ± 20	
D. W. 58, M, W 93.1	Primary gout, tophaceous; benign parotid gland tumor; alcoholism	2.5	14.5	44	6,700	8.0–8.6	317 ± 25	Eleven year history of recurrent gout, 5 attacks per year. BSP retention (27 and 19 %). Developed tophus 1 month following study.
H. H. 62, M, C 102.8	Primary gout, nontophaceous; obesity	25.0	12.2	38	8,850	7.7–8.0	685 ± 82	Attack of gout 1 year and again 1 month prior to study. Unexplained transient hyperglobulinemia of 6 Gm. %. BP, 178/116.
E. H.* 52, M, W 89.4	Primary gout, nontophaceous	5.0	15.7	45	9,100	8.8→4.5	1,054 ± 196	Three attacks of gout, 5, 2, and ½ years prior to study. Probenecid therapy (see footnote).
J. H. 62, M, W	Polycythemia vera; secondary gout, tophaceous	25.0	14.6	45	11,650	7.2–9.6	851 ± 224	Attacks of gout since 1953, responsive to colchicine. Found to have polycythemia vera at time of onset of gout. Treated with phlebotomies and in 1953 with P^{32}. Developed tophi in 1955. In late 1956 marked left shift noted in leukocytes, but marrow was normal. NPN, 49 mg. %.
D. D. 38, F, W	Myeloid metaplasia	20.0	8.3	28	40,000	5.9–6.4	982 ± 88	Three year history of fatigue, and bleeding episodes. Marked splenomegaly. Differential showed left shift with 4% blasts. Bone marrow—myelofibrosis.

* This patient was studied while on probenecid, 1.0 Gm. per day. For some months prior to the study, his serum uric acid level was stable at 4.5 to 4.8 mg. per cent, and his average uric acid excretion during the glycine-1-C^{14} study was similar to pretreatment excretion values (822 and 1,026 mg. per day). He was therefore judged to have mobilized and excreted the excess miscible uric acid and once again to be in a dynamic steady state with respect to uric acid synthesis and excretion.

lected for subsequent recrystallization (8, 18) and C^{14} analysis. The filtrate, containing the urinary purine bases as soluble hydrochlorides, was then treated with ammoniacal silver nitrate and stored in the cold (19). The washed silver salts were decomposed with a minimal quantity of HCl, and the purine hydrochlorides separated from the AgCl precipitate by centrifugation. This silver precipitation step effected a further purification of the purine bases, which were eventually recovered in a solution of HCl of known strength. This solution was diluted to 0.15 N with respect to HCl and was then added to a Dowex-50-H^+ column, 12X, 200 to 400 mesh. In the last two studies (control H. B. and gouty subject H. H.) urinary purines were isolated by preliminary segregation on Dowex-50-H^+ according to Weissmann, Bromberg, and Gutman (20) and eventually also placed on the analytical column in dilute HCl. The analytical column, 2.5 cm. in diameter, contained 150 ml. of resin in acid form,[2] washed with water till free of Cl^-. The sample was washed into the column with 50 ml. of water, and then elution of purines was started with 0.15 N HCl, under 2 to 4 p.s.i. added air pressure. A continuous frontal analysis of the elution of ultraviolet absorbing compounds was obtained with the recording UV scanner. After passage through the quartz cell of the absorption meter, the eluate was collected in 24 ml. fractions.

Uric acid was not retained by the resin, and the small quantities remaining in the sample after the copper isolation procedure were quickly washed through the column. At about tube No. 65 xanthine appeared. As soon as xanthine was removed, the elution system was changed so as to provide an acid gradient for the remainder of the process. This technique is a modification of the stepwise elution procedure described by Abrams and Bentley (21) and Wall (22).[3] A double reservoir was put in place in which the upper flask, a three-liter separatory funnel, containing 2.2 L. of 2.67 N HCl, was arranged to feed into the lower flask, a two-liter bottle with an outlet near its base, containing 2.0 L. of 0.15 N HCl, as the contents of the latter flowed on the column. Mixing was effected within the lower chamber by means of a magnetic stirrer, and the rate of elution was maintained at about 3 ml. per minute by means of 1 to 2 p.s.i. added air pressure. The identity of the compounds comprising the various peaks was established by comparison with known compounds as to elution sequence, spectral characteristics at pH 2, 6 and 9, 250/260 and 280/260 mμ absorption ratios, response to xanthine oxidase (xanthine, hypoxanthine, adenine) or to guanase in the presence of xanthine oxidase (guanine, 1-methylguanine), and migration on two-dimensional paper chromatograms (23).[4]

The fractions representing a single peak were pooled, and analyzed quantitatively. Xanthine and hypoxanthine were analyzed with xanthine oxidase according to Kalckar (14). Adenine was analyzed with xanthine oxidase in the presence of catalase according to Klenow (24). Compound S, separated from adenine on paper in butanol-ammonia (20, 23) and eluted by downward irrigation with 0.01 N HCl, was shown not to be a substrate for xanthine oxidase, so that adenine analyses could be conducted enzymatically on the buffered mixed sample obtained from the column. Guanine was assayed with guanase in the presence of xanthine oxidase according to Kalckar (13). The methylguanine fraction was assayed by ultraviolet absorption alone, since 7-methylguanine, the major constituent, is not responsive to guanase (25) or xanthine oxidase (26). 1-Methylguanine, the other component, is deaminated by guanase (25), and the product, 1-methylxanthine, is a substrate for xanthine oxidase (27). These reactions permitted the estimate that about 10 per cent of the total methylguanine fraction was the 1-methyl isomer. However, since these compounds rarely separated satisfactorily on Dowex-50 or on paper (20, 23), the mixture was treated as a single fraction, and expressed as 7-methylguanine.

The major goal of the present methods was isolation of the purine bases in quantities sufficient for isotope analysis. Therefore, the appropriate fractions were routinely pooled and reduced to dryness *in vacuo* at 30° C. in a rotary evaporator. Thereafter, the residue was redissolved (or suspended) in a few ml. of water and transferred to a 12 ml. conical test tube. Xanthine was purified by solution in 0.5 N NaOH, decolorized with Norit, and reprecipitated with acetic acid. 7-Methyl-8-hydroxyguanine was recrystallized from HCl by addition of water according to Weissmann, Bromberg, and Gutman (28). Hypoxanthine was isolated as the silver picrate and recrystallized from concentrated HNO_3 (29). 7-Methylguanine was precipitated from dilute HCl with ammonium hydroxide at pH 9 and chilling. It was purified by solution in 2 N HCl, decolorized with Norit, and reprecipitated by adjustment of the filtrate to pH 9 with ammonium hydroxide.[5] Adenine was isolated as the picrate

[2] Dowex-50-H^+ resin was prepared by preliminary washing twice each with 2 N NaOH and 4 N HCl, the last HCl wash being performed in the column and continued until ultraviolet absorbing impurities were removed.

[3] Weissmann, Bromberg, and Gutman (20) have also recently developed a modification of the Wall procedure for use in separation of urinary purines. Their method is similar to that described herein, except that stronger concentrations of HCl were employed.

[4] Xanthine, hypoxanthine, guanine, and adenine standards were available. Their spectra, and 250/260 and 280/260 mμ absorption ratios were obtained in HCl eluates from the column, which thereafter served as references for characterizations of peaks from urine samples. 7-Methylguanine was identified by comparison with spectral data published by Weissmann, Bromberg, and Gutman (20). 7-Methylxanthine, 7-methyl-8-hydroxyguanine, 1-methylhypoxanthine, N^2-methylguanine, 1-methylguanine and "S" were identified by their positions on two-dimensional paper chromatograms, and spectral data following elution from these papers, in comparison with published data (20, 23).

[5] The purity of the foregoing compounds was checked frequently during the course of these investigations.

TABLE II

Purity of adenine picrate

Sample	*cpm/mM*
Picrate of "adenine-S mixture"	782
Picrate of pure adenine	706

and recrystallized from 25 per cent acetic acid (30). The purity of the adenine picrate was demonstrated as follows: An adenine picrate sample was redissolved in 0.01 N HCl, and the picrate removed by repeated extractions with ether (31). The aqueous solution was then placed on filter paper as a band, and a descending chromatogram developed with butanol-ammonia. Following drying of the paper, a single band was seen in ultraviolet light opposite an adenine marker, whereas no absorbing material was seen opposite a compound S marker. The adenine was eluted, and its picrate again prepared. The specific activities of the original and final preparations of adenine picrate were in good agreement (Table II). The preparation of the picrate, therefore, was an effective means of separating adenine from compound S for isotope analysis.

Guanine was not present in urine in sufficient quantity to permit isolation as a crystalline product. Therefore, after the quantity of guanine in the column eluate was established by enzymatic analysis (average *ca.*, 0.3 mg.), 2 to 3 mg. of carrier guanine were added, and the solution was reduced to dryness, redissolved in a few ml. of water and guanine isolated by neutralization of the solution with NaOH and chilling (30). In other cases also, where the quantities of purine separated from the column were very small, as in 6 to 12 hour urine samples, small quantities of the appropriate carrier were added.

The crystalline products were sparingly washed with iced water, dried *in vacuo* at 100° C., and transferred to stainless steel planchets as suspensions in acetone or absolute alcohol, dried under an infra-red lamp, and counted in a Robinson gas-flow counter (32) having a background of 4 cpm, and an efficiency of 53 per cent at infinite thinness. Counting was conducted to less than 10 and usually to less than 5 per cent error (33), and all counting values were corrected to a standard mass of 3.3 mg. per 1.54 cm.^{2} surface area by means of self-absorption correction curves. At this mass, the counting efficiency is 26 per cent. Appropriate conversion factors were employed to permit direct comparison of counting values of purine derivatives with free bases.

The values for hypoxanthine and xanthine obtained from the optical density readings of the column eluates agreed with values obtained from analyses with xanthine oxidase (14). Also, the concentrated eluates were shown to yield but one ultraviolet absorbing spot each when checked by two-dimensional chromatography (23). Furthermore, when the crystalline free bases xanthine, 7-methylguanine, and 7-methyl-8-hydroxyguanine were recovered from planchets, redissolved in HCl, and rechromatographed on paper, they appeared pure.

RESULTS

Separation of urinary purines

A typical elution pattern is illustrated in Figure 1. Uric acid was washed off the resin with water. Immediately following the uric acid peak, there appeared a small second peak (a) of unknown identity. At about tube No. 65, xanthine appeared, and thereafter, following the changeover to the gradient elution procedure, the other purine bases appeared sequentially as indicated in Figure 1. The tube number at which a specific purine base appeared seldom varied by more than two or three from that indicated in the figure, in about 120 separate sample isolations. At about tube No. 110, a second unknown appeared (b). Substances a and b exhibit only sweeping end-absorption in the ultraviolet, and do not migrate in the two-dimensional paper chromatogram. Compound S appears to be identical with the compound so labeled by Weissmann, Bromberg, and Gutman (20). Substance c was inconstant. It did not migrate on the two-dimensional chromatogram. When purines are isolated by initial segregation on Dowex-50, the elution pattern from the analytical column does not contain uric acid, peak a or peak c, and small peaks or shoulders representing compounds V and W (20) are found. None of the

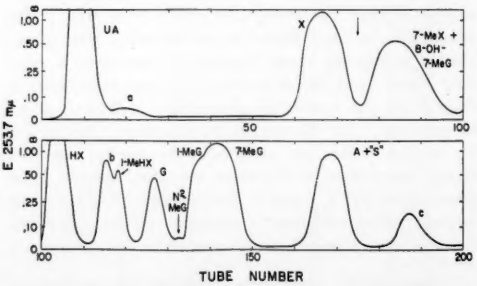

FIG. 1. CHROMATOGRAPHIC SEPARATION OF URINARY PURINES

Urinary purines, isolated by sequential copper and silver precipitations, were separated on Dowex-50-H$^+$ resin by elution with HCl. Initial elution was with 0.15 N HCl. After appearance of xanthine, gradient elution was begun with 2,200 ml. of 2.67 N HCl in the upper reservoir and 2,000 ml. of 0.15 N HCl in the lower reservoir. Fractions equal 24 ml. each.

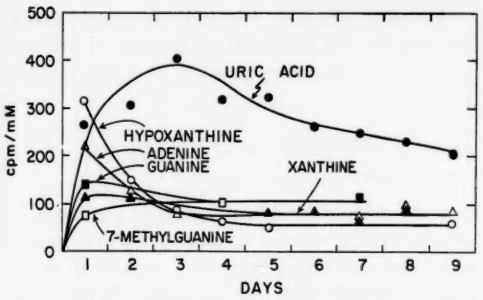

FIG. 2. SPECIFIC ACTIVITY VALUES OF URINARY PURINES OF CONTROL SUBJECT C. L., FOLLOWING ORAL ADMINISTRATION OF 5 μc. OF GLYCINE-1-C^{14}

ultraviolet absorbing compounds gives an orcinol test (34) for pentose, so there is no suspicion that any of the compounds are ribosides. Indeed, ribosides, if present, would in all likelihood have been hydrolyzed during the initial isolation procedure.

Labeling of urinary purines in control subjects

Figure 2 shows the labeling pattern of hypoxanthine, adenine, xanthine and uric acid, and of a few samples of guanine and 7-methylguanine, in control subject C. L. following administration of 5 μc. of glycine-1-C^{14}. Hypoxanthine and adenine were maximally enriched on Day 1, and thereafter rapidly declined to rather low levels of isotope concentration. Xanthine enrichment was also somewhat greater on Days 1 and 2 than on subsequent days, but there was no clear-cut peak on Day 1 as with hypoxanthine and adenine. The scattered samples of guanine and 7-methylguanine [6] similarly do not suggest that either was as highly labeled at any time as were hypoxanthine or adenine. Uric acid attained maximal isotope concentration on Day 3, whereafter a slow decline ensued. Hypoxanthine was the only urinary purine base showing C^{14} concentration greater than that of uric acid on Day 1.

[6] It appears likely that 7-methylguanine is derived from guanine rather than from more complex guanine compounds, since only in the free base form is there a replaceable hydrogen on N-7. Thus, the labeling patterns of 7-methylguanine are believed to reflect those of guanine, and therefore also those of guanylic acid. Data shown in Figure 10, and to some extent Figure 2, support the interpretataions of the significance of 7-methylguanine labeling values, although the interpretations are only inferential.

In order to elucidate more clearly the events occurring immediately following administration of glycine, a second control subject, H. B., was given 25 μc. of glycine-1-C^{14} and fractional urine samples were obtained initially. Uric acid became maximally labeled in the 6 to 17 hour sample (Figure 3), the earliest time a maximum has been observed in a normal subject. However, cumulative incorporation of C^{14} into urinary urate in 2.75 days was 0.064 per cent of the administered dose, an entirely normal value (8). Hypoxanthine enrichment was maximal in the 0 to 6 hour sample and its specific activity was three times that of uric acid of the same specimen, and almost two times the maximal uric acid value, attained during the next 11 hour period. Hypoxanthine enrichment promptly fell to rather low specific activity values. Adenine was also maximally labeled in the 0 to 6 hour specimen and declined in isotope concentration thereafter. In contrast, xanthine and 7-methylguanine showed rather low specific activity values in the 0 to 6 hour specimen, and passed through isotope concentration maxima in the 6 to 17 hour period, declining strikingly immediately thereafter. The parallelisms of the hypoxanthine and adenine curves and of the xanthine and 7-methylguanine curves are to be noted.

These studies strongly suggest that the rapid

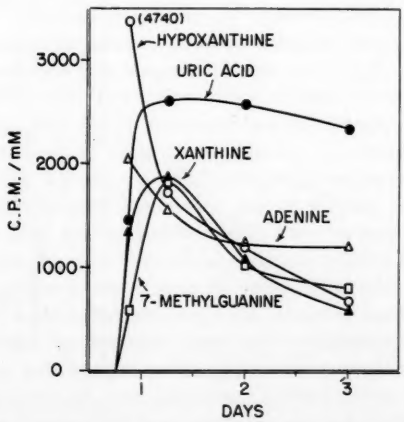

FIG. 3. SPECIFIC ACTIVITY VALUES OF URINARY PURINES OF CONTROL SUBJECT H. B., FOLLOWING ORAL ADMINISTRATION OF 25 μc. OF GLYCINE-1-C^{14}

One 6, one 12, and two 24 hour urine specimens were collected. Note particularly the very high initial C^{14} content of hypoxanthine.

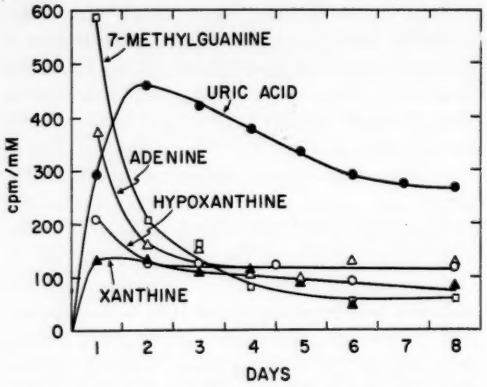

FIG. 4. SPECIFIC ACTIVITY VALUES OF URINARY PURINES OF GOUTY SUBJECT D. W., FOLLOWING ORAL ADMINISTRATION OF 2.5 μC. OF GLYCINE-1-C^{14}

Note the high initial enrichment value of 7-methylguanine.

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synthesis of labeled uric acid from glycine-1-C^{14} in control subjects proceeds via hypoxanthine compounds and probably free hypoxanthine as a major intermediate. The most direct pathway employing known reactions involves stepwise build up of glycine to inosinic acid (29, 35–39), dephosphorylation to yield inosine (39), phosphorolytic cleavage to yield hypoxanthine (40, 41), and oxidation of hypoxanthine to uric acid (14, 27). One would anticipate that enrichment values of xanthine would reflect its role as product of hypoxanthine and precursor of uric acid in this sequence. A possible explanation of the pattern observed is that hypoxanthine once bound by xanthine oxidase is not appreciably displaced from the enzyme until fully oxidized to uric acid, and that urinary xanthine is derived from organs not actively participating in rapid urate synthesis, or perhaps primarily from guanine rather than from hypoxanthine. The early maxima of adenine, 7-methylguanine and xanthine suggest that all of the newly formed nucleotides may be subject in part to cleavage reactions, and that several pathways may contribute to early labeling of uric acid. However, the initial specific activity data suggest that inosinic acid cleavage may normally be quantitatively the most important of these pathways. Thus, the direct synthesis of uric acid in normal man may well involve primarily the same pathway employed by the bird in the formation of uric acid as its major excretory product of waste nitrogen (29, 42).

Labeling of urinary purines in patients with primary gout

Three patients with primary gout were studied, one excreting normal quantities of uric acid in urine, one excreting amounts somewhat in excess of normal, and one excreting decidedly abnormal amounts. In Figure 4 are shown results obtained in gouty subject D. W., who excreted 317 mg. of uric acid daily. The most striking finding of this study was the level of 7-methylguanine enrichment on the first day, which was twice that of uric acid of that day, and one-third higher than the peak uric acid value found on the second day. The enrichment of adenine in the first day sample was also greater than that of uric acid. In contrast to the control studies, the first day hypoxanthine enrichment was of small magnitude in comparison with that of uric acid in this subject, whose fractional incorporation of administered glycine-1-C^{14} into urinary uric acid was about three times normal (8).

In Figure 5 are shown data on E. H., a gouty subject excreting 1,050 mg. of uric acid daily. Uric acid was maximally labeled on Day 1, and the cumulative incorporation of label into urinary uric acid was some four times normal (8). Hypoxanthine labeling was also maximal on Day 1, and its specific activity curve seemed to parallel

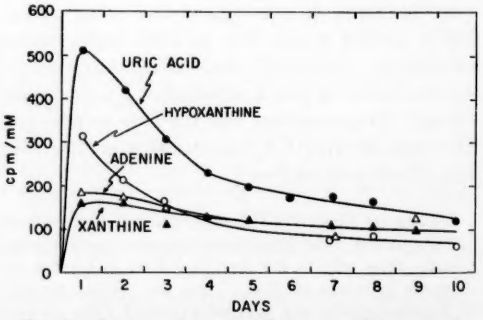

FIG. 5. SPECIFIC ACTIVITY VALUES OF URINARY PURINES OF GOUTY SUBJECT E. N., FOLLOWING ORAL ADMINISTRATION OF 5 μC. OF GLYCINE-1-C^{14}

This subject excreted an average of 1,054 mg. of uric acid daily during this study.

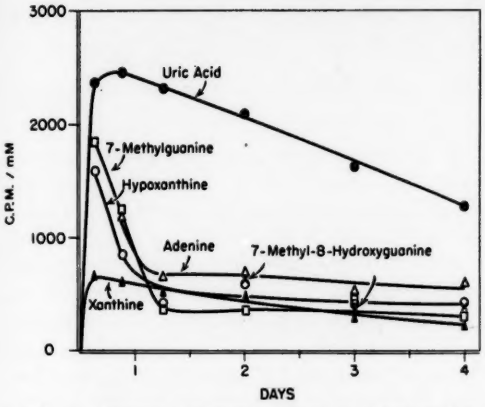

FIG. 6. SPECIFIC ACTIVITY VALUES OF URINARY PURINES IN GOUTY SUBJECT H. H., FOLLOWING ORAL ADMINISTRATION OF 25 μc. OF GLYCINE-1-C^{14}

that of uric acid. Nevertheless, the hypoxanthine specific activity values were at all times considerably less than those of uric acid. It was, however, appreciated that an extremely rapid turnover of the hypoxanthine pool in both gouty subjects D. W. and E. H. might have yielded highly labeled uric acid, as observed, and that the maximal labeling of urinary hypoxanthine might not be discernible within a 24 hour sample. For this reason, a third gouty subject was studied in whom fractional urine samples were obtained during the first day.

Figure 6 shows data obtained on H. H., a gouty subject excreting an average of 685 mg. of uric acid per day. Uric acid enrichment was maximal between 6 and 12 hours following oral administration of 25 μc. of glycine-1-C^{14}, and the cumulative incorporation of C^{14} into urinary uric acid was 0.19 per cent in four days, a value about 2.5 times normal (8). The labeling of other purines was greatest in 7-methylguanine, hypoxanthine, and adenine. However, the shapes of the curves suggest that specific activity values were probably higher during the initial hours than is reflected in the pooled 0 to 6 hour sample. It is to be noted that in this patient, as in patient D. W., the early specific activity values of 7-methylguanine are high compared with results in the two control subjects. In this subject, studied during the waning days of a prolonged attack of acute gouty arthritis, the urinary excretion of 7-methyl-8-hydroxyguanine was about 5 mg. per day, a value two to three times normal (20). Three samples of this purine were successfully isolated, and specific activity values were similar to those of other purines on Days 2 to 4.

These studies suggest that the prompt excessive synthesis of uric acid found in gouty subjects may involve early cleavage of purine nucleotides, as in normal subjects. The high early labeling of 7-methylguanine in two gouty subjects, not found in the control subjects, is of interest. The observation that urinary excretion of 7-methyl-8-hydroxyguanine may be increased during acute gouty attacks (11, 43), demonstrated again in patient H. H., further directs attention toward guanine compounds in this disease.

In order to determine to what extent the 7-methylguanine enrichment pattern might be specific for patients with primary gout, it was decided to conduct similar studies on patients with known overproduction of uric acid involving excessive turnover of nucleoproteins.

Labeling of urinary uric acid in patients with proliferative hematopoietic disorders

Since the quantities of uric acid excreted by patient D. D., a female with myeloid metaplasia, and by patient J. H., a male with polycythemia and secondary gout, were considerably greater than those of control subjects, the C^{14} enrichment data

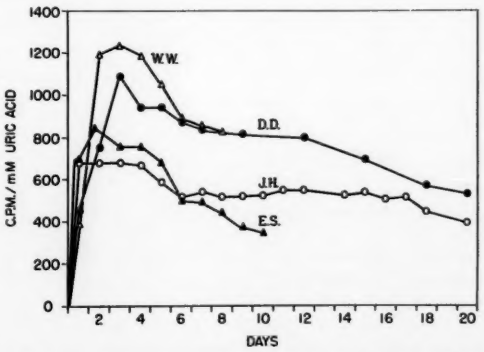

FIG. 7. SPECIFIC ACTIVITY VALUES OF URINARY URIC ACID IN MALE CONTROL W. W., AND A MALE SUBJECT WITH POLYCYTHEMIA VERA AND SECONDARY GOUT, J. H., AND IN FEMALE CONTROL SUBJECT E. S., AND A FEMALE SUBJECT WITH MYELOID METAPLASIA AND HYPERURICEMIA, D. D.

Values have been adjusted to a standard dose of 20 μc. of glycine-1-C^{14}

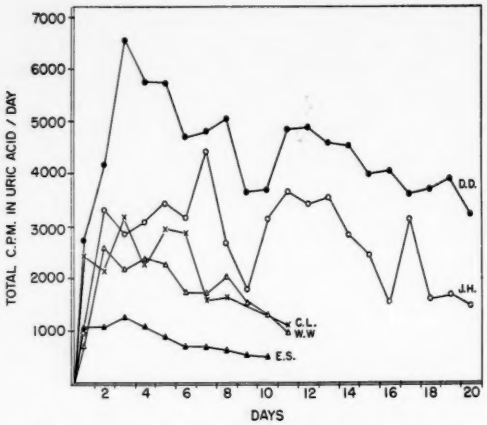

FIG. 8. TOTAL C^{14} IN DAILY URIC ACID SAMPLES

Subjects as described in legend of Figure 7. In addition, data on a second male control subject, C. L., have been included. Note the secondary phases of uric acid enrichment in subjects D. D. and J. H.

are plotted in terms of concentration (Figure 7) and total quantity of C^{14} (Figure 8) in daily samples of urinary uric acid,[7] and in terms of cumulative excretion of C^{14} in uric acid (Figure 9). Although in both patients there is marked early enrichment of uric acid, in neither is the initial (first day) synthesis of uric acid-C^{14} clearly greater than in control subjects, as is regularly found in patients with primary gout (8). In both patients D. D. and J. H. there is, however, evidence for a secondary phase of incorporation of C^{14} into uric acid, maximal on about the eleventh to thirteenth days (Figure 8). This second phase of enrichment is in good agreement with the time of occurrence of isotope maxima in uric acid following glycine-N^{15} administration (9, 10, 12). The purine intermediates involved in these two phases of uric acid production were studied in patient D. D.

Labeling of urinary purine bases in a patient with myeloid metaplasia

In Figure 10 are plotted the specific activity values of adenine, guanine, 7-methylguanine, hypoxanthine, xanthine and uric acid over a 15 day period following administration of 20 μc. of glycine-1-C^{14} to subject D. D. The specific activities of all purine bases exceeded that of uric acid on Day 1, xanthine by about 15 per cent, hypoxanthine by twofold, guanine and 7-methylguanine by more than threefold, and adenine by more than sixfold. There is an immediate sharp decline in specific activity of these bases such that xanthine and 7-methylguanine reach low values by the second day. Minimal values of adenine and hypoxanthine are not reached for five to seven days, and these results are presumably a reflection of the slow rate of turnover of the adenine pool (44). Uric acid reached maximal enrichment on Day 3 and thereafter declined in C^{14} concentration. The secondary phase of uric acid enrichment demonstrated above is now seen to be a reflection of secondary maxima occurring also in the various purine bases. The parallelism of the enrichment curve of 7-methylguanine (and guanine) with that of xanthine, and of adenine with that of hypoxanthine, are worthy of note, as is the lack of a close correlation between the shapes of the hypoxanthine and xanthine curves themselves. These relationships have certain implications regarding precursor-product relationships. Suffice it to say here that Figure 10 is believed to present

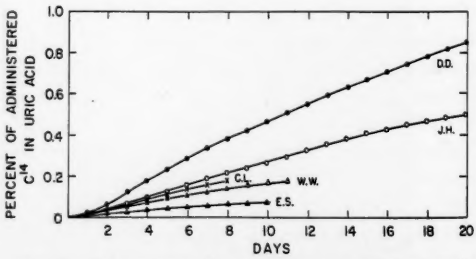

FIG. 9. CUMULATIVE INCORPORATION OF C^{14} INTO URINARY URIC ACID OF SUBJECTS DESCRIBED IN LEGENDS OF FIGURES 7 AND 8

Data are plotted in per cent of administered dose versus time.

[7] Wide fluctuations in uric acid excretion occurred in patients D. D. and J. H. Low outputs on Days 9 and 10 in D. D. and Day 9 in J. H., as well as high outputs on Days 7 and 17 in J. H., have perhaps introduced fortuitous effects in Figure 8. The difference in C^{14} uric acid output between control and experimental subjects is most remarkable on Days 10 and 11. Although the data on control subjects have not been extended beyond 11 days in the present study, other studies have shown that there is no secondary late accentuation of uric acid labeling in control or primary gouty subjects (12) such as has been demonstrated here and elsewhere (12) in patients with myeloproliferative disorders.

evidence for two classes of pathways of uric acid synthesis, differing remarkably in rate, but involving the same intermediates. The rapid process, yielding significant labeling of purine bases on Day 1, is reflected in a rapid enrichment of uric acid. The slower process, yielding secondary maxima in purine bases 7 to 11 days later, is in turn reflected in a second week enrichment peak in uric acid. This late process is quite consistent with known time relationships of ribonucleic acid turnover (45, 46), although other mechanisms, such as reutilization of glycine moieties released from protein, might also be entertained.

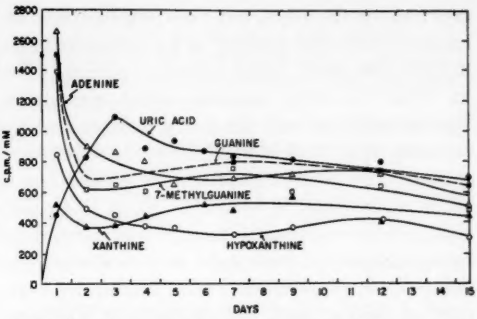

FIG. 10. SPECIFIC ACTIVITY VALUES OF URINARY PURINES IN D. D., A PATIENT WITH MYELOID METAPLASIA WHO RECEIVED 20 μC. OF GLYCINE-1-C^{14} ORALLY

Note particularly the high initial specific activity values of all purine bases and the secondary maxima occurring on Days 7 to 12, illustrating two distinct mechanisms for synthesis of labeled uric acid.

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DISCUSSION

From the knowledge that purine bases are not formed *de novo*, but are rather generated as ribotides (35, 36, 38, 39, 47–49), it follows that the prompt appearance of highly labeled purine bases in urine results from cleavage of newly formed nucleotides. There is evidence for the existence of this mechanism in all subjects of the present study, although there is no way of determining from the data of this paper whether ribotides or deoxyribotides are primarily involved. Insofar as the bases generated are hypoxanthine, xanthine, or guanine, mechanisms exist for their prompt oxidation to uric acid. Hypoxanthine is readily oxidized to uric acid via xanthine by xanthine oxidase (14, 27). Guanine is deaminated by guanase to form xanthine (13), which is then converted to uric acid by xanthine oxidase. Adenine, however, cannot be directly deaminated in man (50) so that free adenine is converted to uric acid only following reincorporation as riboside (40) or ribotide (51) and further metabolism via inosine or inosinic acid (39).

Since it is known that nucleoside phosphorylase attacks inosine and guanosine much more readily than adenosine (40, 41, 52) or xanthosine (53), it might be anticipated that stepwise cleavages of inosinic acid to hypoxanthine and of guanylic acid to guanine, with subsequent conversion of the free bases to uric acid, would constitute major pathways of the shunt mechanism. The isotope data in the control subjects accord with these anticipations. In both subjects the initial high labeling of hypoxanthine provides strong indication that cleavage of inosinic acid constitutes a major pathway for direct synthesis of uric acid. The early labeling of guanine in subject C. L. and of 7-methylguanine [6] in subject H. B. suggest that the guanylic acid cleavage pathway accounts for a smaller portion of the early labeling of uric acid.

In the patient with myeloid metaplasia, the very high specific activity values of guanine compounds, and of adenine, suggest that direct pathways involving nucleotides of guanine and adenine were quantitatively of greater than usual significance. In view of the recognized overproduction of nucleic acids in this subject, the high labeling of bases derived from these nucleotides is not surprising. Weissmann, Bromberg, and Gutman (28) have observed initial high N^{15} labeling of 7-methylguanine, 7-methyl-8-hydroxyguanine and adenine in a patient with polycythemia vera given glycine-N^{15}, so that the isotope relationships observed in subject D. D. may be common in patients with proliferative hematopoietic disorders.

The major goal of this study was the acquisition of information regarding mechanisms by which persons with primary gout synthesize uric acid excessively. Time relationships suggest that overproduction of uric acid by these subjects occurs without the intermediary intervention of nucleic acids (5, 7, 8). The data presented suggest further that rapid synthesis of labeled urate in these patients occurs via nucleotide cleavage pathways, as in normal subjects and patients with myelo-

proliferative disorders, but to a quantitatively excessive extent, and possibly at an accelerated rate, in that even short initial collection periods failed to reveal the true maximal initial enrichment. Furthermore, in both the gouty patients and the subjects with myeloid metaplasia, the labeling of guanine compounds appeared to be greater and to occur earlier than in normals. Since in patients with myeloproliferative disorders a generalized overproduction of nucleotides is of necessity present as an accompaniment of the accelerated turnover of nucleic acids, the similarity of the initial labeling patterns of urinary purines in the patient with myeloid metaplasia and in subjects with primary gout suggests that the patterns observed in primary gout may also be a reflection of overproduction of various nucleotides from glycine and other small molecules. Again, the possibility that deoxyribotides may participate must be kept in mind. At any rate, it appears unlikely that the metabolic error of primary gout consists solely of an accentuation of a single cleavage pathway. The data of the present paper implicate overproduction of uric acid probably via inosinic acid, and abnormal labeling of 7-methylguanine. Recent data from Gutman's laboratory (12, 43) implicate altered excretions of 6-succinoaminopurine (the aglycone of the intermediate ribotide in the inosinic-adenylic acid transformation), and 7-methyl-8-hydroxyguanine (of unknown origin) during the acute gouty attack. Thus, the gouty derangement would appear to be complex, indeed, and probably to involve several compounds at the nucleotide level. It is not possible at present to formulate a hypothesis based on a single missing or deficient enzyme that would adequately explain these findings. It may be, however, that the metabolic defect of primary gout involves primarily a defect in regulation of rates of nucleotide synthesis rather than a specific missing reaction. The findings of Seegmiller, Laster, and Stetten (54) that aminoimidazole carboxamide given concomitantly with glycine-N^{15} reduces the incorporation of N^{15} into urinary uric acid in both normal and gouty persons, but frequently less so in the gouty, indicates that purine nucleotide synthesis in man is subject to regulatory mechanisms which may be defective in primary gout.

Possible reasons for the differences obtained with glycine-N^{15} and glycine-1-C^{14} in studies of uric acid generation in primary gout have previously been discussed (8). It was demonstrated that the quantity of glycine administered as glycine-N^{15} was in excess of tracer levels, and that the magnitude of early incorporation of label into uric acid was percentually low as a consequence. The results obtained in patients with proliferative hematopoietic disorders (D. D. and J. H.) with glycine-1-C^{14} are also different from those reported with glycine-N^{15}. With the N^{15} technique the early phase of isotope incorporation is not as much in evidence, a rather smooth increase in isotope concentration of uric acid usually being seen, reaching a maximum at about 10 to 15 days (9, 10, 12). The studies with tracer doses of glycine-1-C^{14} have, however, shown clearly that there are two major phases to this incorporation process. Although the results of both processes have been interpreted in terms of known reactions, accessory pathways of nucleotide and purine synthesis leading to production of uric acid have not been excluded. The time lapses between peak enrichments of primary precursor purines and uric acid in some studies, *e.g.,* control subject C. L. (Figure 2) and patient D. D. (Figure 10), raise such possibilities. Potential reactions of this type, *e.g.,* involving an intermediary role of uric acid riboside, have been discussed (4). It would seem more likely, however, that these isotope relationships, as well as the lack of conventional precursor product concentration relationships of hypoxanthine, xanthine, and uric acid, or of guanine and xanthine, are manifestations of the complexities of kinetics of body pools of intermediates, and of the limitations of information obtainable from urinary products having diverse origins. Thus these studies, although suggestive of types of mechanisms involved in uric acid synthesis, will have to be supplemented by more detailed studies of reactions conducted in isolated tissue systems. Such studies are now in progress.

SUMMARY

1. Procedures are described for isolation from urine of hypoxanthine, xanthine, adenine, guanine, 7-methylguanine and 7-methyl-8-hydroxyguanine in crystalline form suitable for measurement of C^{14} content.

2. These purine bases have been isolated following administration of glycine-1-C^{14} to control

subjects and patients with primary gout or myeloid metaplasia. Specific activity values have been compared with those of uric acid over variable periods of time in normal subjects and in patients with disorders of purine metabolism.

3. Two major mechanisms exist by which uric acid is generated in man. In addition to the traditional reactions by which catabolism of nucleic acids gives rise to purine bases susceptible of subsequent conversion to uric acid, there appear to be nucleotide cleavage reactions which yield highly labeled purine bases in urine within hours after administration of glycine-1-C^{14}.

4. It is proposed that in normal and gouty subjects the synthesis of *labeled* uric acid from glycine-1-C^{14} occurs primarily via the "shunt" mechanism; in contrast, in the patient with myeloid metaplasia, a second mechanism, presumably that of augmented nucleic acid turnover, accounted for the late (second week) synthesis of considerable additional labeled uric acid.

5. On the basis of the patterns of labeling of urinary purines in patients with primary gout, and a comparison of these patterns with the initial purine labeling pattern of a patient with myeloid metaplasia and generalized overproduction of nucleotides, it is proposed that patients with primary gout may also have faulty regulation of rates of nucleotide synthesis, and that in these patients, in contrast to those with myeloproliferative disorders, excessive purine nucleotide synthesis is followed by rapid conversion of surplus purine bases to uric acid. The data suggest that several purine nucleotides may be involved in the derangement of purine metabolism of primary gout.

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THE MEASUREMENT OF THE PULMONARY DIFFUSING CAPACITY IN THE PRESENCE OF LUNG DISEASE

By D. V. BATES

(From McGill University Clinic, Royal Victoria Hospital, Montreal, Canada)

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Recent work (1–4) has shown that the rate of pulmonary gas diffusion is abnormal in a variety of clinical conditions, and its measurement seems certain to become a necessary part of any complete evaluation of pulmonary function. The various methods of measurement so far described, whether using carbon monoxide or oxygen, are capable of providing a theoretically valid figure for the diffusing capacity in normal subjects, since in them the lung may be regarded as behaving in an approximately uniform manner. A number of papers have been published in which measurements of the rate of gas diffusion in disease have been presented. A steady state Dco technique devised by Filley, MacIntosh, and Wright (5) has been used in a study of a variety of patients with pulmonary fibrosis (3, 6). The oxygen method has been used in studies of sarcoidosis (7), emphysema (2), mitral stenosis (8), and a number of other conditions. The single breath modified Krogh method developed by Ogilvie, Forster, Blakemore, and Morton has been used to study selected cases of a variety of different clinical conditions (9) and one study has been reported in a small group of patients using radioactive carbon monoxide (10). A steady state CO method in which the alveolar CO was calculated from an assumed value of respiratory dead space has been used in an attempt to establish the value of this type of function test in the assessment of emphysema (4), and a modified CO steady state technique has been used to investigate the causes of disability in pneumoconiosis (11).

Patients in whom there is little or no inequality of gas distribution or circulation in the lung do not present a major problem, since in them it can be assumed that during a steady state, a reasonably representative estimate of the mean alveolar tension of CO can be obtained either by measuring the end tidal CO tension, or by calculating alveolar CO tension from the measured values of arterial carbon dioxide tension, as described by Filley, MacIntosh, and Wright (5). When it is known, however, that there is not only unevenness of distribution of inspired gas within the lung, but imbalance between ventilation and perfusion, as in the case of emphysema, the situation is very much more complicated. It cannot be assumed that in such lungs there is any constant ratio between the surface area available for diffusion and the gas volume and ventilation of different parts of the lung. It is immediately clear that in patients in whom CO_2 retention is present and in whom there is a gross unevenness of gas distribution within the lung, the meaning of any single figure for the pulmonary diffusing capacity, however determined, will be questionable. Ideally, the pulmonary diffusing capacity should be the sum of the individual diffusing capacities for each alveolus. When the perfusion, diffusion, and ventilation are varying widely throughout the lung, the true diffusing capacity will be the aggregate sum of a vast number of widely differing numbers. It seems unlikely that such a figure can ever be arrived at, since if carried to its logical conclusion it would involve measurements made on individual alveolae. It is reasonably certain that no overall number for the pulmonary diffusing capacity which is found in these complex disease states will approximate to this ideal and unobtainable figure. The problem, therefore, becomes one of attempted assessment of the meaning of individual diffusion figures, however obtained, and it is the purpose of this paper to discuss the validity of steady state measurements of pulmonary diffusing capacity in lung diseases in which there is known to be gross unevenness in relation to perfusion and ventilation within the lung. Although it is not possible, for obvious reasons, to calibrate any particular method against a theoretical ideal figure, enough data have been collected to permit discussion of the significance of the results found in a number of different diseases. In particular, it is possible to estimate the significance

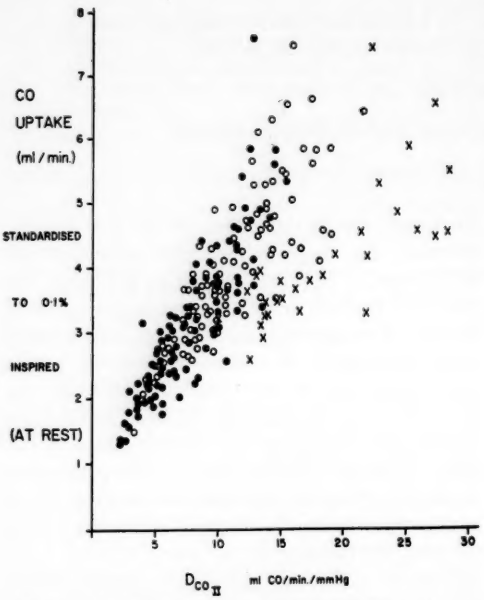

FIG. 1 (A). RELATIONSHIP BETWEEN THE CO UPTAKE STANDARDIZED TO A CONSTANT INSPIRED GAS PERCENTAGE (0.1 PER CENT CO) AND THE PULMONARY DIFFUSING CAPACITY CALCULATED FROM AN END TIDAL SAMPLE (Dco_{II})

Although there is a general relationship between these two observations it is clear that the diffusing capacity could not be adequately calculated from the rate of uptake of carbon monoxide alone. All subjects studied at rest.

× = normal subjects; ○ = patients with normal gas mixing; ● = patients with uneven gas mixing [helium mixing efficiency below 50 per cent (13)].

of the results obtained using a steady state carbon monoxide method, which is undoubtedly the simplest method of measurement to use.

METHODS

The technique used for the measurement of the pulmonary diffusing capacity has already been described (12). The original circuit has been modified by the addition of a photoelectric relay to open the electromagnetic valve at the end tidal sampling position. The light to the photocell is interrupted by a vane in the transparent plastic tube on the inspiratory side of the mouthpiece. An electronic timer is thus actuated by the beginning of inspiration and opens the end tidal electromagnetic valve for any chosen time period. In this way the volume of the end tidal sample can be varied, and is independent of tidal volume.

The subdivisions of lung volume have been measured on a closed helium circuit as previously described (13). The arterial pCO_2 has been calculated from the pH measured on a Beckman pH meter at 37.5° C., and total CO_2 content measured on a Van Slyke apparatus.

A small respiratory circuit was built so that an end tidal sample could be taken during quiet breathing of room air during the simultaneous collection of a resting arterial blood sample. The design of this was exactly similar to the circuit used for the measurement of the pulmonary diffusing capacity, but the end tidal gas was passed through a Cambridge differential catharometer, the calibration of which had previously shown that the CO_2 concentration could be read to approximately 0.05 per cent. The formulae used in the calculations are given in the Appendix.

RESULTS

The results obtained are shown in Figures 1 to 3 and may be conveniently described under various subheadings:

Rest

In a preceding communication (12), it was pointed out that in normal subjects at rest the calculation of a mean alveolar CO using an assumed value of respiratory dead space is liable to be grossly in error. This fact was appreciated by Krogh and Krogh (14), and is the result of the sensitivity of the Bohr equation at low tidal volumes to changes in the assumed dead space value. If, however, the rate of CO uptake in ml. per minute is grossly reduced, as it is in emphysema for instance (15), this error becomes less important, and an estimate of gas diffusion can be made in patients of this type even when an assumed value of dead space is used, provided the limitations of this type of measurement are clearly recognized.

In Figure 1 (a), the CO uptake, standardized to a constant inspired concentration of 0.1 per cent, has been plotted against the Dco_{II} (diffusing capacity calculated from measured end tidal CO concentration). It will be realized that the numerator of a fraction is plotted against the numerator divided by the mean alveolar CO concentration. It is clear from this graph that the resting diffusing capacity cannot be judged with any accuracy from the rate of uptake of CO. A figure of 11 ml. CO per minute per mm. Hg may be taken as aproximately the lower limit of normal for a resting Dco by this technique (5, 12).

On the basis of this figure a resting uptake of less than 2.5 ml. CO per minute at an inspired concentration of 0.1 per cent might be taken to indicate considerable impairment of diffusing capacity at rest. The rate of uptake of CO at rest is, however, greatly influenced by ventilation (15) and thus a demonstrably abnormal diffusing capacity may exist in someone with a rate of uptake of CO as high as about 4.0 ml. CO per minute per mm. Hg. A similar graph [Figure 1 (b)] of the relationship between the fractional alveolar removal of CO, based on an assumed value for the respiratory dead space and the Dco_{II}, shows a wider scatter of results, and there is no reason to suppose that this index offers any advantage over the Dco_{II}.

Exercise

It has been generally realized that when on exercise the tidal volume exceeds about 800 ml., the calculation of concentration of mean alveolar gas from the Bohr equation becomes relatively insensitive to any chosen value for respiratory dead space. In a previous communication (12), it was shown that in young normal subjects, on moderate exertion, the diffusing capacity calculated from an end tidal sample did not differ greatly from the diffusing capacity found if the mean alveolar CO was calculated from an assumed dead space value. It was expected that the good general relationship between the diffusing capacity calculated by either of these techniques would break down in conditions in which the lung was ceasing to behave as a uniform system. Figure 2 shows the relationship between these two measurements on 151 patients suffering from a variety of respiratory conditions and 24 normal subjects. The volume of respiratory dead space has been taken to correspond with the body weight in pounds (16). Every estimate made over an 18 month period has been included in this and subsequent figures. The good general agreement between the Dco_I (calculated from the Bohr equation, using an assumed value of respiratory dead space) and Dco_{II} (calculated from the measured end tidal sample) can be seen. From the point of view of assessment of pulmonary function, it is clear that on exercise either of these methods might be used without much influencing the estimation of degree of abnormality. It is of particular interest that there is no evidence of greater discrepancy between these two calculated values of diffusing capacity in patients known to have uneven gas distribution in the lungs (Figure 2, solid circles) than there is in patients in whom the index of helium mixing was normal (Figure 2, open circles). The general agreement between these two indices does not, of course, indicate that either of them is a correct measurement of the "ideal" pulmonary diffusing capacity. It does mean that under conditions of exercise the mean alveolar CO may either be calculated from an assumed dead space value, or directly measured from an end tidal sample, and that even if gas

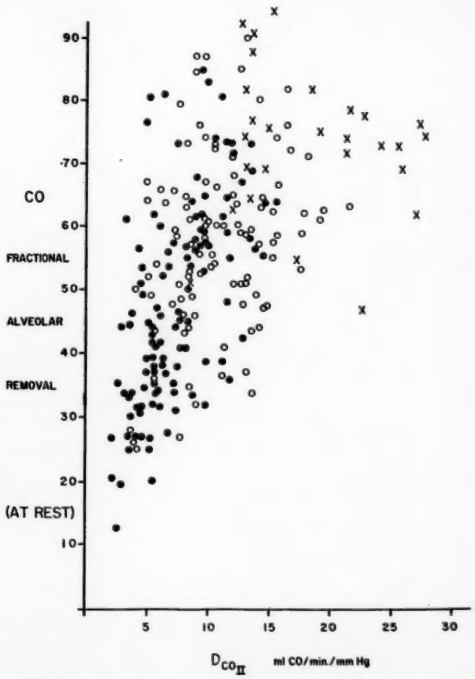

FIG. 1 (B). RELATIONSHIP BETWEEN THE FRACTIONAL ALVEOLAR REMOVAL OF CO AND THE PULMONARY DIFFUSING CAPACITY CALCULATED FROM AN END TIDAL SAMPLE (Dco_{II})

An assumed value of respiratory dead space calculated from the patient's weight has been used in computing the fractional CO removal. All subjects studied at rest. The sensitivity of the calculation of fractional removal to the assumed value of dead space makes this figure of little use in assessment of abnormality.

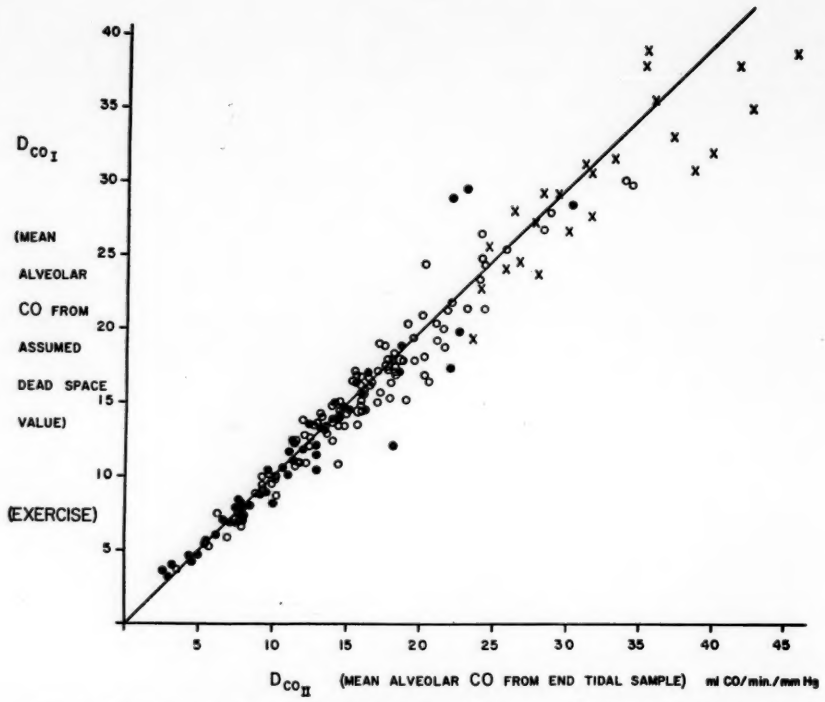

FIG. 2. RELATIONSHIP BETWEEN THE PULMONARY DIFFUSING CAPACITY CALCULATED FROM AN ASSUMED VALUE FOR RESPIRATORY DEAD SPACE (ORDINATE) AND THE PULMONARY DIFFUSING CAPACITY CALCULATED FROM AN END TIDAL SAMPLE (ABSCISSA)

All observations made during exercise, and tidal volume in each case exceeding 800 ml. Results on 24 normal subjects and 151 patients [symbols as in Figure 1 (a)]. The close relationship between the two values for the diffusing capacity can be seen, and in particular there appears to be no evidence that the presence of uneven gas distribution causes any greater discrepancy between these observations than when the gas distribution is within normal limits.

distribution is uneven in the lung it will make very little difference to the calculated diffusing capacity which of these two techniques is used. There are three possible reasons for this. The first, already mentioned, is that if the tidal volume is large, errors in the assumed dead space value have little effect on the calculated mean alveolar CO concentration. The second possible reason for the general agreement shown in Figure 2 is that even in emphysema there may be no gross variation in CO concentration in different parts of the expiration under these conditions. Ogilvie, Forster, Blakemore, and Morton (9) have recently reported that using the single breath Dco technique, the calculated Dco in six normal subjects and two patients with emphysema was very little influenced by whether an "early" or "late" alveolar sample had been taken. The data in Figure 2 support this finding, since if the end tidal CO concentration were to be grossly different from the mean alveolar CO, this discrepancy would show itself by more variation and probably a systematic difference between the ordinate and the abscissa. The third reason why these two differently calculated pulmonary diffusing capacities do not show any wide variation, even in the presence of uneven gas distribution, is that the main determinant of an impaired pulmonary diffusing capacity on exercise, when measured with carbon monoxide and the steady state method, is the grossly impaired rate of uptake of the gas. It is important to remember that the calculated dif-

fusing capacity is the rate of uptake in ml. per minute divided by the mean alveolar carbon monoxide. In patients with impaired diffusion for any reason, the numerator of this fraction, that is, the rate of uptake of the gas in ml. a minute, is the major factor responsible for producing a low figure for the diffusing capacity, and the relative changes in the bottom of the fraction, which is the mean alveolar CO, become decreasingly important.

This is illustrated in Figure 3 (a), which is similar to Figure 1 (a) except that all the observations were made during exercise. The general relationship between these two observations is better during exercise than at rest, and it appears that when the diffusing capacity is significantly impaired, this fact could be detected with fair precision by measuring the rate of uptake of carbon monoxide only under these conditions. For instance, on any exercise it may be taken that

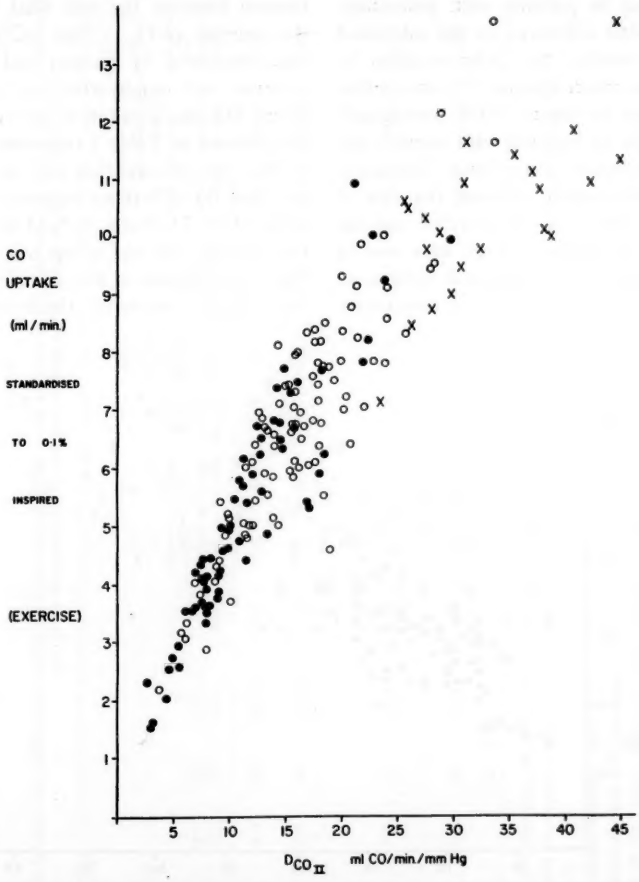

FIG. 3 (A). RELATIONSHIP BETWEEN CO UPTAKE STANDARDIZED TO 0.1 PER CENT INSPIRED AND $D_{CO_{II}}$ CALCULATED FROM AN END TIDAL SAMPLE DURING EXERCISE

Symbols as in Figures 1 and 2. On exercise there is a close relationship between these two variables and an abnormal pulmonary diffusing capacity on exercise could be predicted with fair accuracy from the measured rate of CO uptake alone.

a Dco_{II} of less than 20 ml. CO per minute per mm. Hg is likely to be abnormal (5, 12), and it can be seen from Figure 3 that a rate of uptake of about 6.25 ml. of CO per minute on exercise approximately corresponds with this Dco value. The absolute rate of uptake of carbon monoxide in ml. per minute with a similar inspired concentration of CO was found by Filley and co-workers (5) to vary from 6.8 ml. per minute to 19.5 ml. per minute in the normal subjects studied on exercise. From Figures 2 and 3 it may be concluded, firstly, that in patients with pulmonary disease it makes little difference to the calculated Dco on exercise whether the Bohr equation be used to calculate the mean alveolar CO, or whether an end tidal sample be taken. This correspondence applies equally to patients with normal and abnormal gas distribution in the lung. Secondly, there is a close relationship between the rate of uptake of CO and the Dco_{II} on exercise, and the rate of uptake alone might be used as a test of normality of the lung. The fractional removal of CO calculated on an assumed value of respiratory dead space shows some general relationship to the Dco_{II}, but would not appear to offer any substantial advantage over it [Figure 3 (b)].

Further experimental results are shown in Tables I to III. The data in Table I have been compiled from observations made during simultaneous end tidal CO_2 measurements and arterial puncture. On theoretical grounds, the end tidal CO diffusing capacity (Dco_{II}) is likely to differ from that calculated from the arterial CO_2 by the greatest amount when there is a very large difference between the end tidal CO_2 tension and the arterial pCO_2. This pCO_2 difference has been measured by Saxton and Wolter (17) in patients with emphysema, and a mean value of 8 mm. Hg was found in 11 patients studied. That the patients in Table I represent severe examples of this type of condition may be judged from the fact that the difference between end tidal and arterial pCO_2 (Column 8) was in no patient lower than 18 mm. Hg, and in one was as high as 27 mm. Hg. An estimate of the diffusing capacity at rest immediately preceded these observations, and

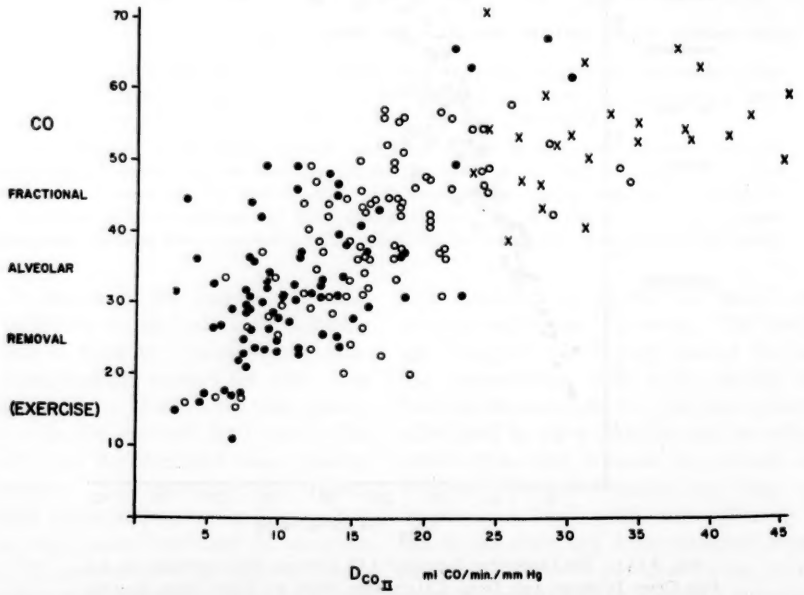

FIG. 3 (B). RELATIONSHIP BETWEEN THE FRACTIONAL ALVEOLAR REMOVAL OF CO AND THE PULMONARY DIFFUSING CAPACITY CALCULATED FROM AN END TIDAL SAMPLE (Dco_{II})—ALL PATIENTS WERE STUDIED IN EXERCISE

The fractional CO removal is in general reduced during exercise, since the level of ventilation increases proportionately more than the increase in diffusing capacity.

TABLE I

Comparison between the pulmonary diffusing capacity calculated from an end tidal CO sample (Column 11a), from the end tidal CO_2 (Column 11b), and from the arterial CO_2 tension simultaneously measured (Column 11c) *

Case†	Diagnosis	1 FRC‡ *liters*	2 ME %§	3 Tidal/vol. *liters*	4 R/min.	5 Vol. of end tidal sample *ml.*	6 Arterial pCO_2 ($PaCO_2$) *mm. Hg*	7 End tidal pCO_2 *mm. Hg*	8 (6–7) Δ $PaCO_2$ and end tidal pCO_2 *mm. Hg*	9 CO uptake *ml./min.*	10 Calc. mean alv. pCO *mm. Hg* via $F_{ET}CO$ (a)	via $F_{ET}CO_2$ (b)	via $PaCO_2$ (c)	11‖ Calc. Dco via $F_{ET}CO$ (a)	via $F_{ET}CO_2$ (b)	via $PaCO_2$ (c)	12 O_2 Hb. satn. %
1	Emphysema	6.4	23	0.765	15	29	46	28	18	3.10	0.43	0.49	0.33	7.5	6.3	9.3	88
2	Alv. hypovent. syndrome	2.0	39	0.334	27	28	55	31	24	4.10	0.335	0.29	0.23	12.8	14.0	17.7	89
3	Emphysema (severe)	3.4	29	0.61	18	25	59	40	19	1.75	0.408	0.422	0.255	4.3	4.15	6.8	78
4	Emphysema (severe)	6.6	36	0.372	29	27	52	25	27	2.35	0.522	0.541	0.262	4.5	4.45	8.95	81

* See Appendix for meaning of symbols used in this table.

† These cases were selected to represent examples in whom there would be a gross difference between the end tidal CO_2 and the arterial CO_2. This difference is shown in Column 8.

‡ FRC = Functional residual capacity.

§ ME % = Helium mixing efficiency per cent, normally above 50 per cent (13).

‖ For discussion of the results shown in this column, see text.

TABLE II

Analysis of the results of measurements of the pulmonary diffusion capacity during maximal exercise in four patients with emphysema and three with proven Hamman and Rich fibrosis

Case	Diagnosis	1 He mixing efficiency %	2 Exercise (flat) *mph*	3 O_2 uptake *liters/min.*	4 Min. vol. *liters*	5 D_{CO_I}*	6 $D_{CO_{II}}$	7 D_{O_2} (1.23 × $D_{CO_{II}}$)	8 Mean gradient ($O_2 \div D_{O_2}$)† *mm. Hg*
1	Emphysema	30	1½	0.94	28.0	12.5	13.9	17.1	55.0
2	Emphysema	25	1½	0.77	22.5	7.1	7.3	8.97	85.8
3	Emphysema	40	¾	0.41	14.7	4.7	4.9	6.04	68.0
4	Emphysema	30	2	0.74	18.2	10.1	10.2	12.5	59.5
5	Diffuse interstitial fibrosis (severe)	49	2	0.58	21.0	4.5	4.6	5.65	102
6	Diffuse interstitial fibrosis (severe)	67	2	1.06	28.6	5.5	6.8	8.36	126
7	Diffuse interstitial fibrosis (severe)	46	2	1.23	32.0	8.7	8.8	10.8	113

* There is no major discrepancy between the D_{CO_I}, calculated from an assumed value of respiratory dead space, and the $D_{CO_{II}}$ calculated from an end tidal sample. All subjects breathing air during these estimations.

† In Column 8 is shown the mean gradient that results from converting the Dco to the D_{O_2}, and dividing it into the oxygen uptake simultaneously measured. Note that the mean gradients so calculated in Column 8 in the cases of Hamman and Rich syndrome are impossibly large. See text for discussion of this finding.

combining the data, the Dco may be calculated three ways: first, by using the measured end tidal CO (Column 11a); secondly, by using the measured end tidal CO_2 in the Filley calculation (Column 11b); and, thirdly, by the Filley calculation using the measured arterial pCO_2 (Column 11c). The results in Column 11a and Column 11b should be the same if the gas analyses are correct, and if there is no gross difference between the behavior of the lung in respect of diffusion and perfusion. It can be seen from Table I that agreement between Columns 11a and 11b is close. It is apparent, however, that there may be differences between the Dco calculated from the end tidal CO or CO_2 (Column 11a and b) and that measured by the Filley method (Column 11c). These are exemplified by Case 4 in which one figure was double the other. Yet in Patient 3, in whom the gas mixing defect is as severe (Column 2), and the pCO_2 differences considerable (Column 8), the difference between the two measured diffusing capacities is much less. The order of difference in these patients, though considerable on a percentage basis, would not produce any great discrepancy in the assessment of pulmonary function, though examples might well be found

TABLE III

Comparison between function test results in six patients with spasmodic asthma and in eight with pulmonary emphysema

Asthma							Emphysema						
Case	Sex	Age	FRC	ME %	MBC (a) or max. midexp. f. r. (b)* (% predicted)	Resting $D_{CO_{II}}$†	Case	Sex	Age	FRC	ME %	MBC (a) or max. midexp. f. r. (b)* (% predicted)	Resting $D_{CO_{II}}$†
1	F	22	3.5	45	61 (b)	17.5	7	M	48	6.4	24	7.5 (b)	7.5
2	M	24	5.4	24	5 (b)	26.1	8	M	52	5.1	50	44 (a)	5.7
3	M	30	3.4	37	21 (b)	13.7	9	M	63	3.2	28	11 (b)	5.9
4	M	42	3.1	15	31 (a)	12.8	10	M	64	5.5	41	4.2 (b)	6.1
5	F	44	3.1	40	40 (b)	22.0	11	M	68	4.7	25	11 (b)	7.5
6	M	48	3.6	50	31 (b)	26.0	12	M	60	3.3	27	15 (b)	6.6
							13	M	69	4.5	22	8 (b)	7.9
							14	M	64	4.7	43	17 (b)	5.7
Mean values		35	3.7	35	31	19.7			61	4.67	32.5	15	6.6

* Per cent predicted maximal breathing capacity or maximal midexpiratory flow rate.

† Although the gas distribution defect, as shown by the mixing efficiency (ME %), and the ventilatory defect as shown by one or other of the ventilatory tests, are similar in these two conditions, the resting $D_{CO_{II}}$ calculated from the end tidal sample is very much lower in emphysema than in asthma. See text for discussion of significance of this difference. Maximal midexpiratory flow rate measured by Leuallan's technique (34).

in which the differences were large enough to influence the assessment made.

From the point of view of interpretation, the difference between these measurements may be summarized as follows:

1. *End tidal Dco.* Provided that the volume of the end tidal sample is controlled, and provided that the patient's respiratory dead space has been flushed, this will represent the Dco of the predominantly ventilated lung.

2. *Dco calculated from arterial pCO.* Since relatively unventilated but perfused lung (areas of potential diffusion) causes a rise in the arterial pCO_2, this Dco may be thought of as including a component of potentially useable lung surface area which may be "available" only in a limited way for purposes of oxygen transfer.

It is known that in emphysema, at the end of a long period of nitrogen washout breathing oxygen, a deep breath will often produce a peak of nitrogen showing that certain parts of the lungs were not being ventilated at all during ordinary quiet respirations (18). The same phenomenon has been demonstrated by pressure volume studies of the lung which suggest that in this disease there is trapped gas in the lung not taking any part of normal ventilatory exchange (19). The diffusing capacity measured from the arterial CO_2 will include a component influenced by such areas of lung, whereas that measured from an end tidal sample will not include any such part. It is obviously impossible to say which is the correct Dco unless it is specified whether one is including unventilated lung in that definition.

A further check on the measured Dco_{II} figure may be obtained by simultaneous estimation of the oxygen uptake and diffusing capacity during exercise. Using the factor of 1.23 to convert the Dco_{II} to the Do_2, the usual equation may be rearranged:

$$\text{Mean gradient (mm. Hg)} = \frac{O_2 \text{ uptake L./min.}}{(Dco_{II} \times 1.23)}$$

(mean alveolar O_2 pressure minus mean capillary O_2 pressure).

When the patient is breathing air (pO_2 equals 140 mm. Hg), the mean gradient cannot possibly be higher than about 90 mm. Hg. Donald, Renzetti, Riley, and Cournand, who have made many indirect measurements of this value for the calculation of the Do_2, find it to average between 40 mm. Hg and 60 mm. Hg (1), and it seems impossible on theoretical grounds that this mean gradient could exceed 90 mm. Hg. When the Dco_{II} and the oxygen uptake are *simultaneously* measured, a falsely low Dco_{II} in respect of the observed oxygen uptake would be revealed by the finding of an impossibly high mean gradient. In a recently completed study (20) of 26 patients with pulmonary sarcoidosis in different stages, the diffusing capacity was measured by the end tidal sampling technique during exercise, and the O_2 uptake was simultaneously estimated. The calculated mean gradients on exercise ranged from 28.4 mm. Hg to 89.0 mm. Hg, with a mean of 49.0 mm. Hg. These patients had no evidence of uneven gas distribution in the lungs. Similar measurements have been recorded by Cugell, Marks, Ellicott, Badger, and Gaensler (3) using the Filley technique and studying patients with pulmonary fibrosis without gas distribution defects. In their group of 23 patients, the mean gradients similarly calculated from the recorded Dco and oxygen uptake range from 24.9 mm. Hg to 88.4 mm. Hg, with a mean of 55.2 mm. Hg. These general figures are remarkably similar and suggest that in this group of diseases it matters little which technique is used to measure the mean alveolar CO. In other exercise studies using end tidal sampling on patients with thyrotoxicosis, anemia, polycythemia, and a wide variety of other clinical conditions, the calculated mean gradients appear to be within reasonable limits (21).

Table II shows this type of calculation in seven patients. The first four were suffering from established emphysema, and the level of exercise they achieved represented nearly the maximum possible for them. All the patients were noted to be very dyspneic after this exercise, and although no attempt was made to train them to even higher rates of effort, it may be taken that they were working nearly at the highest level they could achieve. The diffusing capacity was measured simultaneously with the oxygen uptake. In the final column the calculated mean gradients are shown. It is not, of course, possible to fix an absolute upper limit to the mean gradient under these circumstances, but it seems likely that a mean gradient of 85 mm. Hg, as recorded in the second patient, might be taken as definitely indi-

cating that the Dco as measured must have been too low. The last three patients in Table II were all suffering from a fibrosis of the Hamman and Rich type, the diagnosis in each case being confirmed by lung biopsy. It will be seen that in them there was only minimal defect in gas mixing, and it may be taken that the distribution of alveolar gas was very little impaired in these patients. In spite of this, the diffusing capacity, which in each of these patients was measured twice, when converted to the Do_2 and divided into oxygen uptake, produces a number for the mean gradient which appears to be far higher than it could reasonably be with the patient breathing air. In the case of Patient No. 6, for instance, when the inspired oxygen tension was only 140 mm. Hg, the mean gradient was 126 mm. Hg. There is probably some explanation why this condition gives rise to this aberrant figure on this type of calculation, and it is of interest that it appears to be the only condition capable of doing so. There is no gross disturbance of gas distribution in these subjects, and hence there is no reason to suppose that there is any great discrepancy between the end tidal CO_2 and arterial CO_2. It is possible that there is a CO_2 gradient in this disease between alveoli and pulmonary capillary blood, yet this should not affect the calculated Dco_{II} which, on the basis of end tidal sampling, should still be capable of giving a correct figure for the mean alveolar CO. The difficulty might be arising, however, because the conversion factor from Dco to Do_2 may be incorrect in this disease. Theoretically, the abnormal cells causing the thickening of alveolar walls characteristic of the Hamman and Rich syndrome might be more permeable to oxygen than to carbon monoxide, and if this were the case the error in the calculation lies in the conversion of the Dco to the Do_2. If, in Patient No. 6 of Table II, the conversion factor from Dco to Do_2 should be 1.84 and not 1.23, the mean gradient would be reduced from 126 mm. Hg to 85 mm. Hg, a figure that might be acceptable. A second and more likely explanation is that if the abnormal cells in the lung are metabolizing oxygen to any great extent, then the rate of oxygen uptake as a whole would be an overestimate of the amount of oxygen diffusing through the barriers in the lung. In the case of the three patients in Table II, one would have to postulate that the lung cells were taking up 86, 350, and 313 ml. O_2 per minute to explain the falsely high gradients on this basis, and it seems unlikely that the oxygen uptake of the lung tissue could be as high as these figures. Neither of these explanations can be regarded as satisfactory, and the finding of an apparently absurdly high mean gradient in this condition must be confirmed in similar cases before its significance can be assessed.

In Table III, six cases of asthma are compared to eight cases of emphysema and the striking difference in the Dco's found in these two groups is illustrated. It is possible in asthma to have a considerable mixing defect and ventilatory defect and yet have a normally preserved diffusing capacity at rest (Case 2), and in emphysema there may be fairly good preservation of ventilatory function and a borderline normal figure for evenness of gas mixing, and yet considerable abnormality in the calculated diffusing capacity (Case 8). The clinical selection of these subjects is not a simple matter. The cases of emphysema shown in Table III were clinically advanced cases with constant effort dyspnea and a preceding long history of chronic bronchitis. Previously recorded experience has shown that advanced cases of emphysema may be selected with reasonable certainty by these criteria (4). The patients in the asthma group had all been followed for many years in the allergy department. In all of them there was evidence of seasonal bronchospasm due to allergic sensitivity, and in none was there constant effort dyspnea. This table serves to illustrate the fact that the level of the diffusing capacity found by end tidal sampling with subjects at rest is not inevitably influenced by the evenness of gas distribution, and is certainly capable of discriminating between these two conditions by the difference in diffusing capacity between them. This is well illustrated by a comparison between the second patient and the tenth. With similar functional residual capacities and ventilatory impairment, and the mixing defect greater in the patient with asthma, the Dco_{II} in the asthmatic was 26.1 ml. CO per minute per mm. Hg, and in the patient with emphysema it was only 6.1 ml. CO per minute per mm. Hg. Tomograms of the lungs of these two patients showed a normal small vessel pattern in the asthmatic, and a great loss of peripheral pulmonary vessels in the patient with

emphysema. In spasmodic asthma, if the degree of bronchospasm is so severe that the patient is only ventilating a very small volume of the lung, a slight reduction in diffusing capacity may be found, but it is not reduced to the levels seen in patients with advanced emphysema.

DISCUSSION

The theoretical considerations governing the validity of measurements of the pulmonary diffusing capacity have been discussed in detail by Forster, Fowler, and Bates (22). If the pulmonary diffusing capacity is to be defined as the sum of the diffusing capacities of each alveolus, it is clear that no measurement of it that is "rigorously correct" can be made when there is variation of diffusion within the lung. All contemporary methods of measurement depend on estimating the rate of gas uptake, either over a timed interval of breath holding or during a steady state experiment, and dividing this by a calculated mean pressure of the gas. No "overall" measurement made in this way can claim to necessarily represent the correct overall diffusing capacity of the lungs. The "limitations" imposed on the various methods of measurement may be summarized as follows:

A. Methods using carbon monoxide

(*a*) Uncertainty whether Dco can be converted to Do_2 under all circumstances of disease, particularly in the Hamman and Rich syndrome (see results above).

(*b*) Interference of COHb in the blood (23). Possibility of local back pressure effect in the plasma due to slow passage of CO into the red cell (24).

(*c*) Single breath method (3, 9)

1. Measurement influenced by ratio of diffusion to contained gas volume in the lung (22).
2. Assumption that total rate of uptake of the gas during held breath can be calculated from held portion of gas expired may be false in some cases.
3. Uncertainty whether the lung in a single held breath is typical of the lung during steady state conditions.

(*d*) *Steady state methods*

1. *Filley method* (3, 5, 6). Use of arterial pCO_2 may lead to a higher estimated Dco than is "available" to the patient for oxygen transfer; probably approximates to the highest likely Dco.
2. *End tidal sampling method* (12). This represents the diffusing capacity of predominantly ventilated lung. At rest is bound to be lower than the Filley Dco in the presence of CO_2 retention. A small end tidal sample is essential to avoid dead space contamination which would cause a falsely low figure. On exercise the Dco calculated from the end tidal sample agrees closely with that calculated from an assumed value of respiratory dead space even in the presence of unequal gas distribution in the lung (Figure 2).

(*e*) All steady state methods are influenced by the ratio between the distribution of ventilation and diffusion within the lung (7, 22).

(*f*) $C^{14}O$ method (25) represents a compromise between (*c*) and (*d*) above. Factors influencing it are difficult to assess.

B. Oxygen method (26)

(*a*) Note that this method avoids difficulties of *A* (*a*), (*b*) and (*c*) above.

(*b*) Uncertainty of effect of hypoxia on the rate of diffusion through the lung.

(*c*) Overall measurement may be falsely low under pathological conditions (27).

In view of these many uncertainties it is remarkable that similar results have been reported in different diseases using these different methods. Table IV shows some of the results so far reported in three pathological conditions. It is not possible to make detailed comparisons between the results obtained by different authors, since in many cases inadequate clinical details are provided to enable one to judge the severity of the cases studied. When these problems are remembered, the general agreement shown in Table IV leads to the suggestion that although all methods of measuring the pulmonary diffusing capacity in disease may be subject to error, the

TABLE IV

Comparison between results obtained by different authors of measurements of the pulmonary diffusing capacity in three diseases

1 Disease	2 State	3 No. studied	4 Method*	5 Lowest†		6 Highest†		7 Author reference
				Do_2	Dco	Do_2	Dco	
Chronic respiratory disease emphysema								
	Rest	16	$ssDo_2$	4.0	*3.25*	14.0	*11.0*	(1)
	Rest	57	$ssDco_I$	*4.3*	3.5	*21.0*	17.0	(4)
	Rest	6	He-CO S B	*4.3*	3.5	*43.2*	35.2	(9)
	Rest	35	$ssDco_{II}$	*5.0*	4.1	*14.8*	12.0	(21)
Chronic bronchitis and emphysema								
	Rest	12	$C^{14}O$	*13.5*	11.0	*31.0*	25.0	(10)
Chronic airway disease								
	Exercise	27	$ssDo_2$	11.0	*8.9*	45.0	*36.6*	(2)
Emphysema								
	Exercise	15	$ssDco_{II}$	*5.9*	4.8	*17.2*	14.0	(33)
Pulmonary fibrosis (diffuse interstitial fibrosis)								
	Rest	9	$ssDo_2$	4.0	*3.25*	10.0	*8.14*	(1)
	Rest	2	ssDco (F)	*4.8*	3.90	8.6	7.0	(6)
	Rest	2	He-CO S B	*8.7*	7.1	*12.5*	10.2	(9)
	Exercise	4	ssDco (F)	*15.5*	12.6	*22.1*	18.0	(6)
	Exercise	4	$ssDco_{II}$	*5.6*	4.6	*18.2*	14.8	(Table II)
Sarcoidosis								
	Rest	2	$ssDo_2$	7.0	*5.7*	7.0	*5.7*	(35)
	Rest	8	$ssDo_2$	4.0	*3.2*	23.0	*18.7*	(36)
	Rest	3	$ssDo_2$	4.15	*3.4*	10.9	*8.9*	(27)
	Rest	1	He-CO S B	*4.9*	4.0	*4.9*	4.0	(9)
	Rest	7	$ssDo_2$	14.0	*11.4*	18.0	*14.6*	(37)
	Rest	6	ssDco (F)	*7.2*	5.9	*16.1*	13.1	(6)
	Rest	15	$ssDco_{II}$	*5.05*	4.1	*19.4*	15.8	(20)
	Exercise	3	$ssDo_2$	11.6	*9.4*	23.0	*18.7*	(27)
	Exercise	4	ssDco (F)	*6.6*	5.4	*24.5*	19.9	(3)
	Exercise	15	$ssDco_{II}$	*6.5*	5.3	*19.4*	15.8	(20)

* $ssDo_2$ = Steady state oxygen method.
He-CO S B = Modified Krogh single breath method.
ssDco (F) = Steady state CO technique using arterial pCO_2.
$ssDco_I$ = Steady state CO technique assuming value for respiratory dead space.
$ssDco_{II}$ = Steady state CO technique using measured end tidal CO concentration.
$C^{14}O$ = Radioactive CO method.
† The actual measurement is shown in standard type, and the computed equivalent Dco or Do_2 is shown in italics.

same errors are in general affecting all the methods to a similar degree.

In Table III a comparison was made between the function test results found in spasmodic asthma and emphysema. It is evident from this table that although the ventilatory defect in these two conditions may be very similar, as it was in Patients No. 2 and 10 in Table III, the diffusing capacities as measured by the end tidal sampling technique may be widely different. This indicates almost certainly the preservation of a normal lung parenchyma in asthma on the one hand, and its destruction as the essential lesion of pulmonary emphysema on the other. This differentiation has recently received support from a number of different observations. Gough (28) has stressed the pathological differences between the two conditions, and the radiological differences have been emphasized by Hornykiewytsch and Stender (29) from a study of postmortem specimens. The loss of a normal pulmonary arterial pattern in emphysema has also recently been demonstrated by angiocardiograms (30). The findings shown in Table III are of interest because they illustrate the fact that although in two conditions there may be a gross unevenness of gas distribution and con-

siderable ventilatory defect, yet the diffusing capacity measured by the end tidal sampling technique is able to distinguish between them. It has been suggested (31) that function tests might be combined to make a "discriminant function" capable of a more precise diagnosis of emphysema. This may well be found to be the case; but it is important to recognize the possibility that one function test, in this instance carbon monoxide, may be capable of a far greater discrimination between two conditions such as asthma and emphysema than any combination of other tests.

All those who have used the pulmonary diffusing capacity in the assessment of lung function have become convinced of its value. Cugell, Marks, Ellicott, Badger, and Gaensler (3), and Marks, Cugell, Cadigan, and Gaensler (6) have pointed out the importance of this measurement in the proper analysis of patients with pulmonary fibrosis. The loss of diffusing surface after pneumonectomy has been shown to be one of the factors contributing to residual disability (32). Shepard, Cohn, Cohen, Armstrong, Carroll, Donoso, and Riley (2) have shown that the measurement of the pulmonary diffusing capacity in chronic obstructive disease of the airways puts a different perspective on the syndrome of chronic bronchitis and emphysema, since the loss of lung parenchyma that occurs in emphysema may take place with relatively insignificant bronchitis, and this change may not be evident without a measurement of the pulmonary diffusing capacity. There is also some evidence that the measurement of pulmonary diffusing capacity is of value in assessing the prognosis of pulmonary emphysema (4). In view of this preliminary evidence, it is obviously necessary that continuing critical attention be devoted to the methods used in its measurement.

The use of a "maximal diffusing capacity" introduced by Riley appeared to be a useful concept with the Do_2 technique. It has not been possible, however, to demonstrate a consistent plateau of diffusing capacity with the carbon monoxide methods (12, 24), and it seems likely that the hypoxia involved in the Do_2 method may be responsible for the constant finding of a maximal value with that technique.

There can be no doubt that the steady state carbon monoxide technique is the simplest of all existing methods to use. The single breath CO method requires accurate helium analyses in the presence of varying CO_2 concentrations, and has the additional disadvantage of being inapplicable in a dyspneic patient during exercise. Since the normal subject increases the pulmonary diffusing capacity considerably on exercise, the difference between normal and abnormal lungs is greatly accentuated by exercise studies. All patients should be studied during exercise if possible, since not only are the method errors reduced by the increased tidal volume it produces, but the discrimination between normal and abnormal is sharpened. These advantages have been stressed in a number of recent studies of gas diffusion (2, 3, 20, 32, 33).

SUMMARY

1. Data collected during estimates of the pulmonary diffusing capacity on 151 patients have been analysed to clarify the validity of the end tidal sampling method of measuring the mean alveolar CO concentration in lung disease. It has been shown that during exercise there is good correspondence between the Dco calculated from such samples, and the Dco estimated by using the Bohr equation to compute a value for the mean alveolar CO concentration.

2. On exercise, measurement of the rate of uptake of CO alone would be sensitive enough to use in the assessment of abnormality in pulmonary diffusion. This measurement would not be adequate under resting conditions.

3. Comparisons between the Dco calculated from the arterial pCO_2 and the Dco calculated from the measured end tidal CO concentration show that the former is probably measuring a "highest likely" Dco and the latter probably the Dco of predominantly ventilated lung.

4. Evidence is presented that suggests that the steady state Dco measured during exercise in patients with the Hamman and Rich syndrome of diffuse pulmonary fibrosis cannot be correctly converted to the Do_2 by the use of the usual conversion factor.

5. A comparison of results obtained with the end tidal sampling technique in patients with asthma and emphysema indicates that the use of carbon monoxide in these two conditions enables an estimate to be made of the relative normality of the lung parenchyma.

6. In spite of the many contemporary uncertainties in the measurement of the pulmonary diffusing capacity by any technique, the general form of reported results in three different lung diseases suggests that all methods are giving an approximately similar range of results. This probably indicates that all available methods are being influenced in the same direction by similar causes of error.

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I wish to thank Dr. J. A. P. Pare for his assistance in the collection of arterial blood samples, and Dr. R. V. Christie whose encouragement of this work has made its continuation possible.

APPENDIX

Symbols: D_{co} = Diffusing capacity (CO) in ml. per minute per mm. Hg
P_b = Barometric pressure, mm. Hg
V_T = Tidal volume
R = Respirations per minute
V_D = Dead space (includes instrumental dead space
F_I = Inspired
F_{EX} = Mixed expired } Carbon monoxide concentration ATPD
F_{ET} = End tidal

(*a*) D_{coI} calculated from an assumed value of the respiratory dead space:

$$D_{coI} = \frac{V_T(V_T - V_D) \times R \times (F_I - F_{EX})}{(F_{EX}V_T - F_IV_D) \times (P_b - 47)} \text{ ml./min./mm. Hg.}$$

(*b*) D_{coII} calculated on the assumption that the end tidal sample represents the mean alveolar tension:

$$D_{coII} = \frac{V_T \times R \times (F_I - F_{EX})}{F_{ET} \times (P_b - 47)} \text{ ml./min./mm. Hg.}$$

(*c*) Fractional alveolar removal was calculated as follows: Fractional alveolar removal

$$= \frac{V_T \times R \times (F_I - F_{EX})}{F_I \times R \times (V_T - V_D)} \times 100\%.$$

(*d*) $D_{co}(F)$ was calculated using the simultaneously measured arterial pCO_2 to compute the mean alveolar CO tension as described by Filley, MacIntosh, and Wright (5).

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HEPATIC WEDGE PRESSURE, BLOOD FLOW, VASCULAR RESISTANCE AND OXYGEN CONSUMPTION IN CIRRHOSIS BEFORE AND AFTER END-TO-SIDE PORTACAVAL SHUNT [1, 2]

By ALAN G. REDEKER,[3] HERMAN M. GELLER, AND TELFER B. REYNOLDS

(From the Department of Medicine, University of Southern California, School of Medicine, and the Los Angeles County Hospital, Los Angeles, Calif.)

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Surgical decompression of the portal system by construction of an anastomosis between the splenic and left renal veins or between the portal vein and inferior vena cava is an effective treatment for portal hypertension in cirrhosis. Shunts of this type have been shown to be remarkably effective in the prevention of recurrent variceal bleeding (1, 2). In view of the known high incidence of repeated and eventually fatal hemorrhages in those cirrhotics who have already had one episode of variceal bleeding, there is little question concerning the indication for a shunt in such patients, providing they have sufficient hepatic reserve to withstand the immediate trauma of surgery.

With improvement in surgical technique and patient selection, the previously formidable mortality from these operations has been greatly reduced. Consideration can now be given to extending the indication for shunt surgery to patients with demonstrable varices which have not yet ruptured (3). In debating the merits of "prophylactic shunts," we need both statistics concerning the life expectancy and probability of variceal rupture in these patients under conservative therapy, and information concerning the changes in hepatic hemodynamics and metabolism that might result from the various shunting procedures.

The available data bearing on the latter problem are limited and are concerned largely with the changes after splenorenal shunt. Bradley, Smythe, Fitzpatrick, and Blakemore, using the standard bromsulfthalein method to measure liver blood flow, found an average fall of 22 per cent in five patients after this operation (4). Hepatic oxygen consumption did not appear to be decreased. Nardi, using a less widely accepted technique for liver blood flow determination (the disappearance rate of radioactive colloidal chromic phosphate), noted a fall in six of nine patients after splenorenal shunt (5). The mean change in the nine patients was a fall of 24 per cent.

In the end-to-side portacaval shunt, the portal vein is ligated and divided, and its distal end is implanted into the vena cava. Diversion of portal blood from the liver is therefore complete, and hepatic hemodynamic changes would be expected to be even greater than after a splenorenal shunt. Published studies on hemodynamic changes are limited to three cases included in the report of Bradley, Smythe, Fitzpatrick, and Blakemore (4), and in these three, hepatic blood flow fell an average of 36 per cent. In the clinic from which our patients are drawn, end-to-side portacaval shunt is the procedure of choice for the relief of portal hypertension in cirrhosis (6). In 10 such patients, we have made pre- and postoperative comparisons of the standard liver function tests, and the data obtainable from hepatic vein catheterization, namely, hepatic blood flow and oxygen consumption, wedged hepatic venous pressure, and postsinusoidal hepatic vascular resistance. This communication details our findings and discusses their implications on the therapy and pathologic physiology of portal hypertension.

MATERIALS AND METHODS

Ten patients with end-to-side portacaval anastomoses were the subjects of the study. All were chronic alcoholics and in each, a surgical liver biopsy confirmed the diagnosis of cirrhosis. Esophageal varices were visualized by X-ray in seven cases and by esophagoscopy in

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three. Bleeding from ruptured varices had occurred in all patients except one (J. J.). He had splenomegaly, leukopenia and large esophageal varices demonstrable by X-ray and esophagoscopy. The patients were studied prior to and again following the shunting procedure, as soon as the clinical condition permitted. In two subjects (J. J. and T. S.), the preoperative studies were made on the day of surgery, just prior to the operation. The postoperative studies were made within two months of surgery in eight subjects, three and one-half months after surgery in one (C. M.) and nine months after surgery in another (J. R.).

Hepatic vein catheterization was performed by the usual technique (7). All patients were fasting at the time of the study. Wedged hepatic vein pressure (WHVP) was recorded with the catheter tip wedged into and occluding a small hepatic venous radical (8). The pressure levels reported are net pressures computed by subtracting the measured inferior vena caval pressures from the measured wedged pressure. The use of vena caval pressure as a reference point eliminates errors due to elevation of right atrial pressure, or to inaccurate positioning of the zero point of the pressure recorder (9). Whenever possible, WHVP was recorded from more than one site in the liver to guard against inaccuracy occasioned by incomplete wedging of the catheter. If different levels of WHVP were encountered at different sites, the highest pressure was utilized. In all the preoperative and in nine of the postoperative studies, WHVP, hepatic blood flow, and oxygen uptake data were obtained with the catheter in the right hepatic lobe. However, in Patient R. R., the postsurgical data were obtained with the catheter in the left lobe, since it was impossible to enter a right hepatic vein sufficiently deep to preclude mixing of vena caval and hepatic venous blood during sampling.

With the catheter deep in a hepatic vein, the estimated hepatic blood flow (EHBF) was determined by the bromsulfthalein (BSP) method (7). After a primer dose of from 40 to 60 mg. of BSP was given intravenously, a dilute BSP solution was infused at a given rate by a constant infusion pump. An amount of BSP primer necessary to produce a plasma concentration of approximately 1.5 mg. per cent was estimated from a previously performed 30 minute BSP test (5 mg. per Kg.), and from the patients' body size. An infusion rate was then chosen (usually from 2.0 to 2.5 mg. per minute) which, it was anticipated, would not exceed the liver's ability to keep the plasma BSP levels constant. Simultaneous samples of femoral arterial and hepatic venous blood were taken at approximately 5 minute intervals beginning 20 minutes after injection of the BSP primer. BSP concentrations in the serum were determined colorimetrically after the method of Gaebler (10). The hepatic plasma flow was computed from the hepatic removal rate of BSP and the peripheral arterial-hepatic venous BSP concentration difference. EHBF was calculated from this value and the arterial hematocrit. The rate of BSP infusion (mg. per minute) was corrected for the rate of change of BSP concentration (ΔBSP) in the arterial plasma by the formula of Bradley, Ingelfinger, Bradley, and Curry (7), and the resultant value was taken to be the hepatic removal rate of BSP (^{R}BSP). The validity of the values for hepatic blood flow obtained in this study was determined by the relationship of the rate of change of the arterial BSP concentration to the hepatic removal rate of BSP. It is felt that the correction applied for the rate of change of the arterial plasma BSP levels is, at best, an approximation, since the plasma volume is estimated from a nomogram. Therefore, as this correction factor becomes large in proportion to the value for hepatic BSP removal, the calculation of EHBF becomes progressively less reliable. Thus, values for ^{R}BSP of less than 1.5 mg. per minute were not considered acceptable in this study unless the rate of change of the arterial BSP concentration was less than 0.00026 mg. per ml. per minute. In the postoperative study of Patient C. W., for example, the ^{R}BSP was small, 1.44 mg. per minute. However, ΔBSP was only 0.00020 mg. per ml. per minute and the EHBF was therefore considered valid. We do not feel that the use of BSP extraction percentage, ^{E}BSP (arterial-hepatic venous BSP difference divided by the arterial BSP concentration), as the criterion for the validity of the hepatic blood flow determination is satisfactory, since this technique automatically eliminates the calculation of high hepatic blood flows if they do exist. As an example of this, in Patient C. M.'s preoperative study, the BSP extraction was only 10 per cent at a plasma BSP level of 2.20 mg. per cent, yet the rate of rise of BSP concentration in the arterial blood was relatively small (0.00016 mg. per ml. per minute), and the ^{R}BSP fairly large (3.28 mg. per minute). By our criteria, this would be a valid estimation of EHBF, whereas it would be unacceptable by the criteria suggested by Bradley, Ingelfinger, Bradley, and Curry (7).

The values for hepatic resistance (HR) were determined by dividing WHVP by EHBF and multiplying the quotient by a factor of 100 to bring the values near unity. If WHVP equals hepatic "sinusoidal" pressure, then HR represents the postsinusoidal vascular resistance.

Simultaneous samples of femoral arterial and hepatic venous blood were obtained anaerobically in heparinized syringes immediately following each BSP sample. Arterial and hepatic venous blood oxygen content was determined by the method of Van Slyke and Neill (11) in Patients R. R., C. M., T. S., J. R., M. L., and J. J., preoperatively, and in R. R., C. M., T. S., C H, and J J., postoperatively. In all other instances, the oxygen content was computed from the blood oxygen saturation (determined with a Waters-Conley oximeter) and blood hemoglobin values.

In the preoperative studies splanchnic oxygen consumption (SOC) was computed from the arterial-hepatic venous oxygen difference and the hepatic blood flow. SOC includes both hepatic oxygen consumption (HOC) and the oxygen uptake of the viscera drained by the portal vein. This latter component of the total SOC is relatively small (see below). Postoperatively HOC was

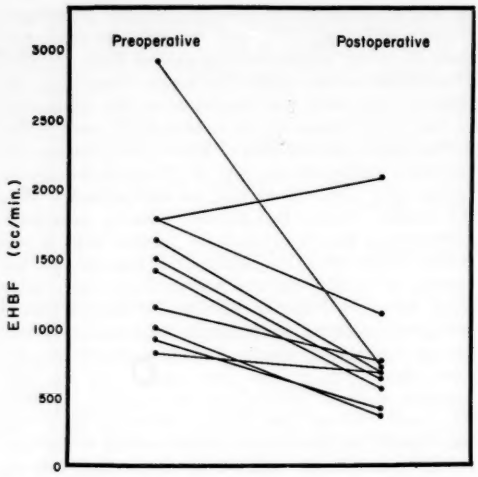

FIG. 1. EFFECT OF PORTACAVAL SHUNT ON ESTIMATED HEPATIC BLOOD FLOW

calculated from the product of the arterial-hepatic venous oxygen difference and the hepatic blood flow. The value no longer includes oxygen uptake of the other abdominal viscera since there is no portal blood entering the liver.

RESULTS

Estimated hepatic blood flow (EHBF)

The EHBF preoperatively ranged from 815 to 2,910 ml. per minute, and averaged 1,490 (±182)[4] ml. per minute. After the shunting operation, the hepatic blood flow ranged from 365 to 2,080 ml. per minute with a mean value of 800 (±93) ml. per minute (Table I, Figure 1).

In 9 of the 10 patients, the EHBF decreased following surgery, while in one instance (Patient T. S.), it increased 16 per cent. The percentage decrease in flow ranged from 15 to 75 per cent, and the average change in the EHBF following the portacaval shunt was a decrease of 46 per cent. In Patient R. R. the preoperative value for the EHBF was unusually high (2,910 ml. per minute). If we were to exclude this value from the results for the EHBF, the adjusted mean preoperatively would be 1,335 (±111) ml. per minute and the percentage decrement following surgery would be 40 per cent. However, since the maximum rate of change of the arterial plasma BSP concentration during that measurement was only 0.0002 mg. per ml. per minute, the EHBF value, in accord with the aforementioned criterion, was considered valid.

It should be pointed out that there are several potential sources of error in the measurement of the EHBF by the bromsulfthalein method, some of them undoubtedly operative in our patients. If, as reported (12), the plasma volume is greater than normal in cirrhosis, then the correction for the changing plasma BSP level will be larger than calculated. This will result in the EHBF being overestimated when plasma BSP levels are rising, and being underestimated when they are falling. Fortunately, in most instances in our patients, plasma BSP levels rose comparably in the preoperative and postoperative studies so that any error introduced by low estimates of plasma volume would tend to be systematic and not contributory to differences between pre- and postoperative flows. Only in Patients M. S. and C. H. were plasma BSP levels rising during the preoperative flow determination, and falling during the postoperative measurement. Since the rate of change of plasma BSP concentration was not excessive in either of these patients, the potential error in the EHBF calculation attributable to incorrect estimation of plasma volume is not excessive. In Patient M. S., for example, if plasma volume is

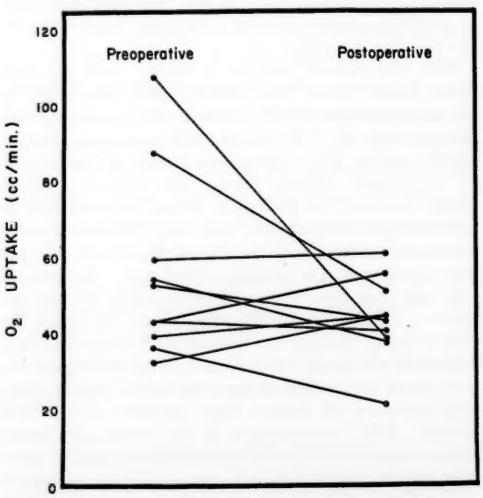

FIG. 2. EFFECT OF PORTACAVAL SHUNT ON HEPATIC OXYGEN UPTAKE

The preoperative figures refer to total splanchnic oxygen uptake, and the postoperative figures to hepatic oxygen uptake.

[4] Standard error of the mean.

actually 50 per cent greater than has been assumed, the preoperative EHBF is 90 ml. per minute too high, and the postoperative value 40 ml. per minute too low.

The influence of the level of arterial BSP concentration on the estimation of hepatic blood flow is a matter of debate. Sherlock, Bearn, Billing, and Paterson felt that arterial BSP levels below 1 mg. per 100 ml. resulted in falsely high EHBF because of the increased importance of extrahepatic BSP removal (13). Bradley, Ingelfinger, and Bradley, on the other hand, has disagreed with this contention (14). Our own opinion is that the arterial BSP concentration is not a critical factor in the measurement of the EHBF, and that extrahepatic removal of BSP is important only when it represents a significant fraction of the BSP removal rate (*i.e.*, when the latter is small). Nevertheless, it would be preferable to make comparative pre- and post-operative EHBF measurements with the same arterial BSP levels, and we were unable to accomplish this in all of our patients.

In some of our patients there was a considerable variation in hemoglobin levels between the preoperative and postoperative EHBF measurements. Other things being equal, a fall in hemoglobin would be expected to cause a rise in the EHBF. In Patients M. S. and M. L., the rise in hemoglobin at the time of the postoperative measurement may have contributed to the observed fall in EHBF. On the other hand, postoperative declines in hemoglobin in Patients C. M., T. S., and J. R. may have minimized the fall in the EHBF in these cases. Since the mean preoperative hemoglobin level of 10.9 Gm. per 100 ml. differs little from the mean postoperative value of 11.0 Gm. per 100 ml., it is assumed that the net effect of the changes in hemoglobin level on the EHBF is insignificant.

Oxygen consumption

Preoperatively, the average value for SOC was 55.5 (±7.4) ml. per minute, while the mean HOC postoperatively was 43.8 (±3.2) ml. per minute (Table I, Figure 2). On the average, postoperative HOC was 21 per cent below preoperative SOC, being lower in six cases and higher in four. This difference is of doubtful significance, statistically. If the data for Patient R. R. are excluded because of marked variation from the mean of the remaining results, preoperative SOC averages

TABLE I

Hepatic vein catheterization data before and after end-to-side portacaval anastomosis *

Subject	Days from surgery		P_{BSP} (mg./100 ml.)		ΔBSP (mg./ml./min.)		R_{BSP} (mg./min.)		E_{BSP} (%)		EHBF (ml./min.)		WHVP (mm. Hg)		H. R. (arb. units)		Art. O_2 content (ml./100 ml.)		H. V. O_2 content (ml./100 ml.)		Art.-H. V. O_2 diff. (ml./100 ml.)		O_2 uptake (ml./min.)	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
R. R.	27	40	1.23	1.49	+0.00020	+0.00016	3.19	3.28	17.1	53.0	2,910	710	14	11	0.48	1.26	15.6	16.2	11.9	10.7	3.7	5.5	107.7	39.0
C. M.	2	107	2.29	1.35	+0.00016	+0.00014	3.28	2.44	10.8	23.0	1,790	1,105	17	13	0.95	1.18	14.7	11.5	12.3	7.8	2.4	3.7	42.9	40.9
T. S.	0	21	1.32	1.24	+0.00026	+0.00012	2.95	2.58	18.9	13.7	1,790	2,080	17	10	0.95	0.48	11.8	8.9	8.5	6.0	3.3	2.9	59.0	60.4
J. R.	49	259	0.85	0.84	−0.00008	+0.00009	2.69	1.74	23.6	41.7	1,630	695	21	13	1.29	2.16	12.3	10.3	6.9	2.9	5.4	7.4	88.0	51.3
M. S.	23	66	0.88	1.36	+0.00018	−0.00015	3.02	2.93	30.6	47.0	1,500	630	22	11	1.46	1.74	7.0	13.5	3.5	6.7	3.5	6.8	52.5	42.8
M. L.	55	54	1.25	2.07	+0.00013	+0.00020	1.99	2.31	15.2	37.6	1,410	570	21	12	1.49	2.11	8.2	18.6	5.4	10.8	2.8	7.8	39.5	44.5
C. W.	22	55	0.76	1.48	−0.00007	+0.00020	2.40	1.44	40.8	18.2	1,150	755	15	12	1.31	1.59	12.5	13.8	9.7	7.9	2.8	5.9	32.2	44.5
J. A.	22	19	0.96	1.46	+0.00004	+0.00023	2.55	1.81	36.5	51.7	1,020	365	18	9	1.76	2.46	11.3	13.7	7.7	7.8	3.6	5.9	36.7	21.5
C. H.	21	15	0.59	1.40	+0.00006	−0.00026	2.46	2.78	65.6	77.1	920	410	20	9	2.12	2.20	15.6	15.4	9.7	6.2	5.9	9.2	54.2	37.7
J. J.	0	21	0.99	1.34	0.00000	+0.00015	2.70	2.33	51.2	36.5	815	690	19	13	2.33	1.89	13.8	12.7	8.5	4.6	5.3	8.1	43.1	35.9
Mean											1,493	801	18	11	1.41	1.70					3.8	6.3	55.5	43.8
S. E. of mean											±182	±93	±0.6	±0.3	±0.16	±0.13					±0.3	±0.5	±7.4	±3.2
S. E. of the difference between means											204		0.6		0.20						0.57		8.0	

* Abbreviations: P_{BSP} = arterial plasma bromsulfalein concentration; ΔBSP = rate of change of plasma level of BSP; R_{BSP} = hepatic removal rate of BSP; E_{BSP} = hepatic BSP extraction percentage; EHBF = estimated hepatic blood flow; WHVP = wedged hepatic venous pressure; H. R. = hepatic postsinusoidal vascular resistance.

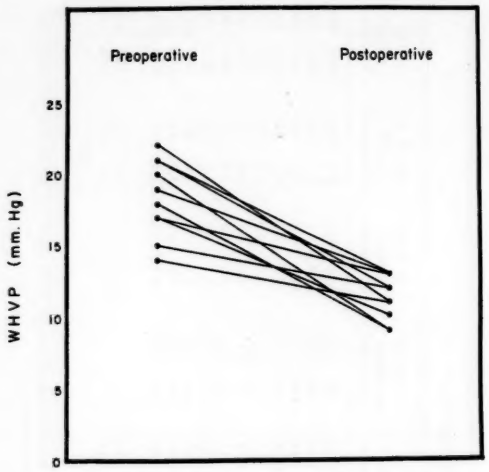

FIG. 3. EFFECT OF PORTACAVAL SHUNT ON WEDGED HEPATIC VENOUS PRESSURE

49.7 (±5.0) ml. per minute, and the percentage decrement following the shunt is 11 per cent.

In 9 of the 10 patients, the arterial-hepatic venous oxygen difference increased after portacaval anastomosis, averaging 3.8 (±0.36) volumes per 100 ml. before and 6.3 (±0.58) volumes per 100 ml. after surgery. The increase in this value following surgery was due to a decrease in hepatic venous oxygen content in all but three of these nine patients. In these three patients (M. S., M. L., and J. A.) there was a higher hepatic venous oxygen content after surgery because of a postoperative rise in hemoglobin, but the arterial-hepatic venous oxygen difference was still increased. The arterial-hepatic venous oxygen difference was slightly decreased postoperatively in one patient (T. S.), and in this patient the EHBF increased slightly.

Wedged hepatic vein pressure (WHVP)

Preoperatively, the WHVP was elevated in all patients, and ranged from 14 to 22 mm. Hg and averaged 18 (±0.63) mm. Hg. Normal values in our laboratory are from 2 to 6 mm. Hg. The WHVP decreased in every instance postoperatively, falling on the average to 11 (±0.31) mm. Hg and ranging from 9 to 13 mm. Hg (Table I, Figure 3). This represented a mean fall in the WHVP of 38 per cent as compared to the mean decrease in the EHBF of 46 per cent. The largest decrements in the WHVP (Patients M. S., J. A., and C. H.) were associated with postoperative decreases in the EHBF of considerable magnitude (50 per cent, 50 per cent, and 55 per cent, respectively), and in the group as a whole, the fall in the WHVP was correlated with the decrease in the EHBF.

Hepatic resistance

The values for postsinusoidal hepatic resistance, expressed in arbitrary units, ranged from 0.48 to 2.33 preoperatively and from 0.48 to 2.46 postoperatively (Table I, Figure 4). The mean values were 1.41 (±0.16) before, and 1.70 (±0.13) after surgery. In a limited number of patients with no evidence of hepatic disease, we have obtained values of from 0.1 to 0.35 for this measurement.

DISCUSSION

In the end-to-side portacaval anastomosis, the portal venous blood is totally diverted from the liver into the inferior vena cava, and the hepatic stump of the portal vein is ligated. Although means for direct measurement of the portal blood flow are not available, an estimate of the value in cirrhosis can be obtained from the difference in hepatic blood flow before and after an end-to-side shunt. The hepatic hemodynamic changes observed following this procedure can be as-

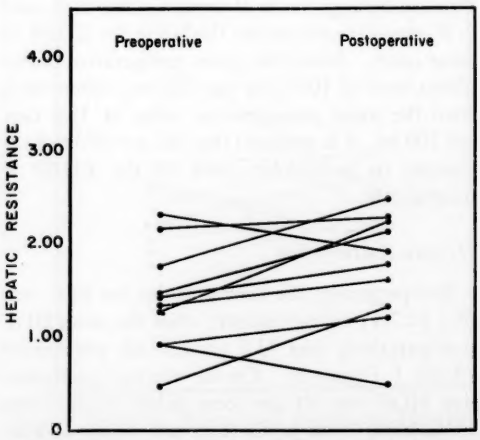

FIG. 4. EFFECT OF PORTACAVAL SHUNT ON CALCULATED HEPATIC POSTSINUSOIDAL VASCULAR RESISTANCE (ARBITRARY UNITS)

cribed only to exclusion of the portal blood from the liver, plus possible readjustments in hepatic arterial inflow. If, following the shunt, no significant change in hepatic arterial flow occurs, then the fall in the EHBF (averaging 46 per cent) presumably represents the portal venous component of the preoperative total liver blood flow. On the other hand, a postoperative compensatory increase in hepatic arterial inflow would modify the fall in total liver blood flow, and imply an even larger magnitude to the portal venous component than the observed 46 per cent. The observed fall in the EHBF, averaging 690 ml. per minute in our 10 cases, must, therefore, be considered a minimal estimate of the size of the portal blood flow. It appears that a very significant portion of the total blood flow of the liver is provided by the portal vein, even in patients with advanced cirrhosis and extensive collateral circulation. This agrees with the findings of Myers who, by administering sodium sulfathiazole orally and comparing simultaneous sulfathiazole levels in hepatic venous and abdominal collateral vein blood, estimated that portal flow ranged between 75 per cent and 25 per cent of the total liver blood flow in three patients with advanced cirrhosis (15). The hypothesis that only a small amount of portal venous blood actually enters the cirrhotic liver, the major portion circumventing it through collateral venous channels, is not compatible with our finding since, under these circumstances, an end-to-side portacaval shunt would be expected to have little or no effect on total liver blood flow. Moreover, if the abnormal hepatic arterial-portal venous communications, which have been described in human cirrhotic livers (16), were significant factors in the genesis of portal hypertension, retrograde flow in the portal vein might be expected to occur, and portal ligation incident to the shunt could actually increase the total liver blood flow.

In the fasting cirrhotic patient, the oxygen content of portal blood is only 1 to 2 volumes per 100 ml. less than arterial blood (4, 17). One might, therefore, expect the large drop in portal blood flow, following a portacaval shunt, to critically reduce the oxygen supply to the liver. Superficially, the 21 per cent difference between preoperative SOC and postoperative HOC would suggest that such a reduction has occurred. However, it must be recalled that in the preoperative patient, the product of the total hepatic blood flow and the arterial-hepatic venous oxygen difference represents the total splanchnic oxygen consumption. Because of the small arterial-portal venous oxygen difference, splanchnic oxygen consumption exceeds the hepatic oxygen consumption by a few ml. per minute. After ligation of the portal vein, only hepatic arterial blood perfuses the liver, and the same calculation gives a value for true hepatic oxygen consumption. The 21 per cent difference between preoperative SOC and postoperative HOC is, therefore, largely, if not entirely, due to inclusion of nonhepatic splanchnic oxygen uptake with the former. Further evidence against a fall in hepatic oxygen uptake is provided by the consistent increase in arterial-hepatic venous oxygen difference, seen postoperatively in all nine patients in whom the EHBF fell. It seems likely that the liver, by extracting increased amounts of oxygen from the arterial blood, is able to maintain its oxygen supply even in the face of a marked fall in total liver blood flow. Of course, since these studies were performed in the fasting state, they may not depict accurately the situation during peak metabolic demands on the liver. In addition, if there were any hepatic cells supplied only by branches of the portal vein, their oxygen supply would be expected to be compromised by a portacaval shunt.

Even though hepatic oxygen uptake is not reduced, the question nevertheless arises concerning what detrimental effects such a striking decrease in liver blood flow might have on a liver whose function has already been impaired by cirrhosis. Table II contains the results of certain standard hepatic function tests performed within a few days of the preoperative and postoperative catheterizations. It is apparent that there are no significant differences in these tests after the shunt, in spite of the magnitude of the decrease in liver blood flow. In a number of other patients whom we have observed following portacaval anastomosis, the standard tests of hepatic function have not been significantly altered except during a 4 to 12 week postoperative period when moderate jaundice and a decrease in serum albumin are the rule. We have not felt this temporary decline in liver function to be out of proportion to that observed in any patient with severe cirrhosis following a

TABLE II

Hepatic laboratory tests before and after end-to-side portacaval anastomosis *

Patient	Preoperative	Postoperative	Time from surgery
R. R.	A/G 4.0/2.0 II 20 BSP 14%	A/G 4.2/3.4 BSP 29%	6 weeks
C. M.	A/G 4.2/3.6 II 7 BSP 24%	A/G 3.4/3.8 BSP 24%	15 weeks
T. S.	A/G 3.7/2.2 II 7 BSP 20%	A/G 3.6/2.0 II 8 BSP 18%	3 weeks
J. R.	A/G 3.3/2.0 II 8 BSP 14%	A/G 3.5/2.5 II 23 BSP 21%†	34 weeks
M. S.	A/G 4.1/1.8 II 13 BSP (less than 10%)	A/G 3.9/3.2 BSP 15%	10 weeks
M. L.	A/G 3.4/2.2 II 22	A/G 3.5/2.8 II 7 BSP 30%	8 weeks
C. W.	A/G 3.3/2.5 II 17 BSP 21%	A/G 3.5/3.0 II 6 BSP 16%	8 weeks
J. A.	A/G 3.6/1.7 II 10 BSP 25%	A/G 3.4/3.2 II 15 BSP 30%	3 weeks
C. H.	A/G 4.0/2.3 II 9 BSP 10%	A/G 3.5/2.7 II 13 BSP (less than 10%)	2½ weeks
J. J.	A/G 3.6/3.6 BSP 19%	A/G 2.7/3.6	3 weeks

* Abbreviations: A/G = Albumin-globulin ratio (Gm. per 100 ml.); II = Icterus index; BSP = BSP retention after 30 minutes (5 mg. per Kg.).
† BSP retention after 45 minutes (5 mg. per Kg.).

major surgical trauma rather than to the drop in liver blood flow.

Of course, diversion of the portal blood from the liver may have more subtle effects on liver function than are measured by the standard tests. For example, "episodic stupor" has been observed to have its onset after portacaval shunt (18, 19). Although it appeared in 2 and possibly 3 of the 10 cases included in this report (Patients J. A., J. R., and J. J.), it has not been a frequent complication of the operation in our experience. These three cases afford us an opportunity to observe any possible relationship between the functional disturbance and magnitude of the postoperative fall in liver blood flow. Although Patients J. R. and J. A. both had large percentile falls in their EHBF, there is no clear cut difference in this respect between these two and the remainder of the group. Patient J. J. actually had a relatively minor change in his EHBF, but it is questionable whether he fits into the category of "episodic stupor." When last seen, he had apparently been showing mental deterioration for approximately two months, and we were unable to determine if it was in any way episodic.

The WHVP was significantly elevated in all patients prior to the portacaval shunt. By this technique the occluding tip of the catheter is, in effect, extended via a static column of blood along the hepatic venule in the direction of the portal system. Presumably, this static column of blood extends no further towards the portal system than the nearest site of freely anastomosing vessels. This site of anastomosis can be termed the "sinusoid," whether one accepts the cord theory or the plate theory of liver anatomy. In cirrhotics with portal hypertension, the WHVP is elevated to a level approximately 20 per cent below portal venous pressure (20). Therefore, the area between the catheter tip and the "sinusoid" can be said to include the major share of the vascular resistance in the liver (excluding presinusoidal hepatic arterial resistance).

Figure 5 is a diagrammatic representation of hepatic circulation, designed to clarify the information provided by the wedge hepatic venous pressure data. In Figure 5a, values for pressure and flow in the hepatic artery, portal vein, and hepatic vein have been assigned commensurate with the catheterization findings in the 10 patients in this paper. Pressure at S is that obtained by wedging the catheter into an hepatic venule (WHVP). The value given for portal vein pressure is based on the assumption that the WHVP is 80 per cent of the portal vein pressure (20). The three resistances R1, R2, and R3 can be calculated by dividing the pressure gradients by the appropriate blood flows and multiplying by 100 to give values (in arbitrary units) of 9 (R1), 0.65 (R2), and 1.21 (R3).

Figure 5b illustrates comparable values for the normal liver. These are necessarily gross approximations, since we have even less data in the normal liver than in the cirrhotic concerning

sinusoidal pressure and the relative contributions of hepatic artery and portal vein to the total liver blood flow. With the values as assigned, R1 is 17; R2, 0.30; and R3 0.33 units. Clearly, R2 and R3 are increased in cirrhosis. If the values for blood flow given in Figure 5b are even gross approximations of the true values, then the major increase in resistance in cirrhosis is on the hepatic venous side of the sinusoid (R3). This is in keeping with the anatomical evidence represented by Kelty, Baggenstoss, and Butt (21).

Although the WHVP fell in all patients after the portacaval shunt, it still remained abnormally elevated. This continued elevation is to be expected if there is no change in intrahepatic vascular anatomy with the operation. In Figure 5c, are charted the mean postoperative values for the blood flow and WHVP in our 10 subjects. R3 is now 1.38, not significantly different from the preoperative level. If the postsinusoidal vascular resistance remains unchanged after the shunt, then the sinusoidal pressure should drop in proportion to the fall in total blood flow. Comparison of the decrements in the WHVP and EHBF in the 10 individual patients indicates that, in general, the two variables did fall proportionately after the portacaval shunt and that the calculated postsinusoidal vascular resistance did not change markedly.

The continued elevation of the WHVP after portacaval shunt does not mean persistence of portal hypertension, since the portal vein has been disconnected from the liver and the WHVP no longer bears any relationship to portal pres-

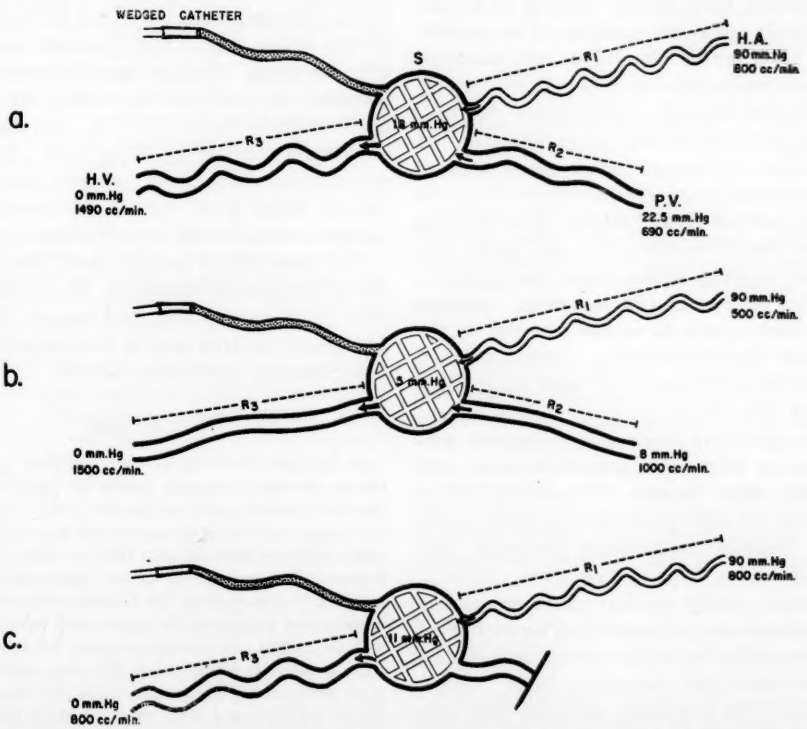

FIG. 5. DIAGRAMMATIC REPRESENTATION OF HEPATIC CIRCULATION SHOWING BLOOD FLOW AND MEAN PRESSURE IN HEPATIC ARTERY (H. A.), PORTAL VEIN (P. V.), HEPATIC VEIN (H. V.), AND SINUSOID (S)

Resistances are indicated by R1, R2, and R3. a, cirrhotic liver; b, normal liver; c, cirrhotic liver after end-to-side portacaval shunt.

sure. The elevated WHVP simply reflects the unchanged hepatic vascular resistance. Portal vein pressures actually fall to insignificant levels after end-to-side portacaval anastomosis, as indicated by direct measurements at surgery and by postoperative catheterization of the portal vein itself, directly through its anastomosis to the inferior vena cava (17).

The therapeutic implications of the above-noted hemodynamic changes should be considered. The volume of portal blood flowing through the liver in patients with advanced cirrhosis and portal hypertension is surprisingly large. Though the end-to-side portacaval shunt has proven to be eminently satisfactory for the prevention of bleeding from esophageal varices, it has the disadvantage of diverting this portal blood from the liver. Although our data do not demonstrate any deterioration in function tests, or any lowering of hepatic oxygen uptake after the operation, it is possible that the reduction in blood flow may interfere with hepatic regeneration or affect other functions that we cannot measure. Consequently, the end-to-side portacaval shunt may eventually prove not to be the best surgical approach to the problem of portal hypertension.

The splenorenal shunt, judging from the data of Bradley and co-workers (4), and Nardi (5), causes less lowering of liver blood flow, probably because of a smaller anastomotic orifice. Pressure in the portal system is seldom lowered to the same degree that it is following a portacaval shunt and, since the portal vein is still open, some portal blood may still perfuse the liver. Unfortunately, there is a significant incidence of recurrent variceal bleeding following splenorenal shunt (2), presumably either because of thrombosis of the anastomosis, or inadequate decompression of the varices. Therefore, as presently performed, splenorenal shunt has definite drawbacks.

Two other possible surgical approaches to portal hypertension may be worth further evaluation. One is arterialization of the stump of the portal vein after end-to-side portacaval shunt. This is feasible (22) but technically difficult. The other is creation of a side-to-side portacaval shunt with an anastomotic orifice of limited size designed to relieve, but not entirely eliminate, portal hypertension and thus preserve some portal blood flow through the liver.

SUMMARY

Measurements of the estimated hepatic blood flow, arterial-hepatic venous oxygen difference, hepatic oxygen consumption, and wedged hepatic venous pressure were made in 10 patients before and after an end-to-side portacaval anastomosis. The hepatic postsinusoidal vascular resistance (the ratio of wedged pressure to hepatic blood flow) was determined pre- and postoperatively. Hepatic function, estimated from the usual laboratory tests, was compared before and after the shunt.

Estimated hepatic blood flow fell in 9 of the 10 subjects following the shunt. In the group as a whole, blood flow fell from 1,490 ± 182 ml. per minute to 800 ± 93 ml. per minute, a mean percentage decrease of 46 per cent. Hepatic oxygen consumption was essentially unchanged after the surgery. Arterial-hepatic venous oxygen difference increased, averaging 3.8 ± 0.36 volumes per 100 ml. before and 6.3 ± 0.58 volumes per 100 ml. after the shunt. Wedged hepatic venous pressure decreased in every instance, falling on the average from 18 ± 0.63 to 11 ± 0.31 mm. Hg, a mean percentage decrease of 38 per cent. The nearly proportional decrease in wedged pressure and hepatic blood flow resulted in essentially unchanged values for the hepatic vascular resistance.

The large fall in hepatic blood flow after the shunt presumably represents the portal component of the preoperative total hepatic blood flow. In spite of the large drop in flow, hepatic function tests were not significantly altered.

CASE REPORTS

R. R. (*#1560–837*), a 51 year old white male with a history of heavy alcoholic intake for many years, was admitted to the hospital on March 6, 1955. Hematemesis and melena had occurred earlier that day and the hemoglobin level on entry was 8.0 Gm. per cent. Emergency treatment included the use of the Sengstaken-Blakemore tube and 10 transfusions. At that time there was hepatomegaly, and the tip of the spleen was palpable. There was no ascites. A few days later, laboratory studies were as follows: icteric index, 20 units; cephalin-cholesterol flocculation, 0; serum protein, 6.0 Gm. per cent (A/G, 4.0/2.0); and BSP retention of 14 per cent after 30 minutes. Needle biopsy of the liver revealed portal cirrhosis. X-ray examination showed large esophageal varices. Hepatic venous catheterization was performed on March 30, 1955. On April 27, 1955, an end-to-side portacaval anastomosis was made; portal venous pressure fell from 340 to 200 mm. saline. Liver biopsy again

revealed portal cirrhosis. Convalescence was uneventful, and he left the hospital 10 days after the surgery. Hepatic venous catheterization was repeated on June 7, 1955; at that time moderate hepatosplenomegaly persisted and laboratory studies showed: serum protein, 7.6 Gm. per cent (A/G, 4.2/3.4); and BSP retention of 29 per cent after 30 minutes. X-rays on July 7, 1955, and on February 14, 1956, showed persistence of the esophageal varices, although greatly diminished in size. He has done well since the shunt, and has returned to full activity.

C. M. (*#632–014*), a 48 year old Mexican woman with a history of chronic alcoholism for many years, was admitted to the hospital on September 16, 1954, because of sudden onset of hematemesis and melena beginning two days before entry. Frequent episodes of epistaxis had occurred during the previous three years, and a clinical diagnosis of cirrhosis was made four months before admission when she complained of recurrent mild epigastric discomfort. Hepatosplenomegaly was noted on admission. There was no ascites or icterus. X-ray examination revealed esophageal varices. Hepatic venous catheterization was performed on October 27, 1954. Laboratory studies at this time were as follows: icterus index, 7 units; serum protein, 7.8 Gm. per cent (A/G, 4.2/3.6); and BSP retention of 24 per cent after 30 minutes. An end-to-side portacaval anastomosis was performed on October 29, 1954. Liver biopsy at operation revealed portal cirrhosis. She recovered uneventfully and X-ray study on January 10, 1955, failed to demonstrate any varices. Postoperative hepatic venous catheterization was done on February 16, 1955, at which time laboratory studies showed: serum protein, 7.2 Gm. per cent (A/G, 3.4/3.8); and BSP retention of 24 per cent after 30 minutes. She has done fairly well since the shunt, except for an episode of mild pancreatitis with elevation of the serum amylase in May, 1956. There has been no further gastrointestinal bleeding.

T. S. (*#1523–196*), a 45 year old white woman with a history of chronic, severe alcoholism, was admitted to the psychiatric unit in September, 1954, for delusions and hallucinations. Hepatomegaly, spider angiomata and icterus were noted, and she was transferred to the medical service. On November 2, while in the hospital, massive hematemesis and melena occurred. Bleeding ceased with the use of the Sengstaken-Blakemore tube but was followed by the appearance of ascites, increasing icterus, oliguria and coma. There was gradual recovery complicated by three small hematemeses. Esophageal varices were not demonstrated by X-ray examination, but esophagoscopy revealed large varices in the distal one-third of the esophagus. The ascites and icterus slowly disappeared. On January 5, 1955, hepatic venous catheterization was performed and a few hours later that day, an end-to-side portacaval anastomosis was performed. Laboratory studies prior to surgery revealed: icterus index, 7 units; cephalin-cholesterol flocculation, 2+; serum protein, 5.9 Gm. per cent (A/G, 3.7/2.2); and BSP retention of 20 per cent after 30 minutes. Liver biopsy at operation showed portal cirrhosis, and the portal vein pressure was 370 mm. saline. Except for transient icterus, the postoperative course was uneventful and hepatic venous catheterization was repeated on January 26, 1955. Laboratory studies at this time showed: icterus index, 8 units; cephalin-cholesterol flocculation, 0; serum protein, 5.6 Gm. per cent (A/G, 3.6/2.0); and BSP retention of 18 per cent after 30 minutes. X-ray examination in April, 1955, again failed to demonstrate esophageal varices. Esophagoscopy has not been repeated. She had had persistent mild ankle edema, but has otherwise been well to date.

J. R. (*#1542–751*), a 55 year old white man with a history of alcoholism for many years, entered the hospital on August 8, 1955, for fever and abdominal pain. Pyelonephritis was diagnosed and treated. During investigation of the abdominal complaints, X-ray studies revealed esophageal varices. Physical examination disclosed hepatosplenomegaly, mild ascites, spider angiomata and "liver palms." There was also a history of heart failure of unknown etiology, controlled with digitalis for the past three years. On October 19, 1955, he suffered a small hematemesis. Hepatic venous catheterization was done on November 1, 1955. Laboratory studies at this time revealed: icterus index, 8 units; serum proteins, 5.3 Gm. per cent (A/G, 3.3/2.0); and BSP retention of 14 per cent after 30 minutes. An operation was recommended, but refused by the patient. Hematemesis recurred on November 26, 1955, requiring the use of the Sengstaken-Blakemore tube and was followed by a transient period of coma. An end-to-side portacaval anastomosis was finally performed on December 20, 1955. Portal venous pressure fell from 350 to 210 mm. saline. Liver biopsy showed portal cirrhosis. The immediate postoperative period was complicated by a thrombophlebitis of the right leg and a pulmonary embolism. While receiving heparin therapy, severe bleeding from the surgical incision occurred requiring many transfusions. Disorientation and a "flapping" tremor were noted frequently during this period. He slowly recovered and did well until August 23, 1956, when a small hematemesis occurred. X-ray examination failed to demonstrate either esophageal varices or an ulcer. The liver remained enlarged, but the spleen could no longer be felt. There was mild ankle edema but no ascites. Laboratory studies at this time showed: icterus index, 23 units; serum proteins, 6.0 Gm. per cent (A/G, 3.5/2.5); and BSP retention of 21 per cent after 45 minutes. Hepatic venous catheterization was repeated on September 4, 1956. There has been no further hemorrhage but he has since suffered repeated periods of mental deterioration associated with gross "flapping" tremor.

M. S. (*#1296–971*), a 48 year old white woman, was hospitalized on June 10, 1955, for hematemesis and melena. She had used alcohol excessively for many years, but none in the past four years. Cirrhosis was diagnosed in 1952, during an episode of ascites, edema and jaundice. She had suffered hematemesis in September, 1954, and again in March, 1955. X-ray studies then failed to dis-

close the source of the bleeding. On admission, there was hepatosplenomegaly, but no ascites. The hemorrhage ceased with the use of the Sengstaken-Blakemore tube. Laboratory studies revealed: icterus index, 13 units; serum protein, 5.9 Gm. per cent (A/G, 4.1/1.8); and normal BSP retention. Hepatic venous catheterization was done on June 28, 1955. Hematemesis recurred on three occasions during the next few days, and was accompanied by transient icterus. An end-to-side portacaval anastomosis was done on July 22, 1955, with a fall in portal venous pressure from 350 to 225 mm. saline. Liver biopsy showed portal cirrhosis. The postoperative course was complicated by mild icterus and a parotitis requiring surgical drainage. She continued to improve and there was no further hemorrhage, although hepatosplenomegaly persisted. The postoperative catheterization was performed on September 27, 1955. Laboratory studies at this time were: serum protein, 7.1 Gm. per cent (A/G, 3.9/3.2); and BSP retention of 15 per cent after 30 minutes. X-ray studies revealed no evidence of esophageal varices. She began heavy drinking in December, 1955, became deeply jaundiced and expired in hepatic coma on April 5, 1956. Postmortem examination revealed cirrhosis, a functioning end-to-side portacaval anastomosis, and no evidence of esophageal varices.

M. L. (*#1328–681*), a 49 year old Mexican man, had been a chronic alcoholic for many years. From 1952 until December, 1954, ascites had appeared from time to time, responding fairly well to diuretics and sodium restriction. Abdominal paracentesis was done twice in 1953. A sudden hematemesis required hospital admission on December 12, 1954, and a Sengstaken-Blakemore tube was passed with control of the hemorrhage. Ascites and hepatomegaly were present at this time. On January 11, 1955, hepatic venous catheterization was done. Laboratory studies at that time showed: serum proteins, 5.6 Gm. per cent (A/G, 3.4/2.2); icterus index, 22 units; and prothrombin time, 68 per cent. X-ray examination in January, 1955, showed large esophageal varices. The formation of ascites gradually ceased, and an end-to-side portacaval anasomosis was done on March 9, 1955, with portal venous pressure falling from 440 to 150 mm. saline. Splenomegaly was noted at operation, and liver biopsy revealed portal cirrhosis. Except for mild transient jaundice, the postoperative course was uncomplicated, and he left the hospital in two weeks. A repeat hepatic venous catheterization was done on May 3, 1955. There was no ascites or icterus at that time, and laboratory studies showed: serum proteins, 6.3 Gm. per cent (A/G, 3.5/2.8); icterus index, 7 units; and BSP retention of 30 per cent after 30 minutes. There was no evidence of esophageal varices on X-ray examination. He has remained free from jaundice or ascites since that time and there has been no gastrointestinal bleeding.

C. W. (*#1086–949*), a 47 year old white man, was admitted to the hospital on December 20, 1955, following sudden hematemesis and melena earlier that day. There was a history of heavy alcoholic intake for 25 years. Transient ascites and jaundice had occurred in 1948 and again in 1952. At the time of entry to the hospital there was hypotension, the blood hemoglobin was 7.5 Gm. per cent, and hepatomegaly was noted. Bleeding promptly ceased with the use of the Sengstaken-Blakemore tube. Esophageal varices were subsequently demonstrated by both esophagascopy and X-ray examination. Laboratory studies on December 28, 1955, revealed: serum proteins, 5.8 Gm. per cent (A/G, 3.3/2.5); icterus index, 17 units; and BSP retention of 21 per cent after 30 minutes. Hepatic venous catheterization was done on January 10, 1956. At operation on February 2, 1956, chronic cholecystitis and cholelithiasis were discovered in addition to cirrhosis. A cholecystectomy and an end-to-side portacaval anastomosis were performed. Portal venous pressure fell from 360 to 150 mm. saline. His recovery was uneventful, without the appearance of icterus or coma, and complicated only by a curious neuritis of the left foot. The postoperative catheterization was done on March 27, 1956. Laboratory studies at this time showed: serum proteins, 6.5 Gm. per cent (A/G, 3.5/3.0); icterus index, 6 units; and BSP retention of 16 per cent after 30 minutes. Esophageal varices were still demonstrable on X-ray, although markedly reduced in size, on May 31, 1956. There has been no hemorrhage or jaundice since the shunt and he has remained fairly well.

J. A. (*#1620–577*), a 65 year old Mexican male, was admitted to the hospital on November 20, 1955, following a massive hematemesis. There was a history of heavy alcoholic intake for more than 20 years. One month prior to admission, he had entered another hospital for hematemesis and melena and had received two transfusions. An X-ray study there was reported to have shown a prepyloric ulcer and antacid therapy had been recommended. Peptic ulcer symptoms were denied. He continued to have melena, and an emergency laparotomy was done on November 22, 1955. A large, nodular liver and a large spleen were found. Duodenotomy revealed no evidence of an ulcer. An omental vein pressure was elevated (approximately 250 mm. saline). A liver biopsy showed portal cirrhosis. Laboratory studies after operation revealed: serum proteins, 5.3 Gm. per cent (A/G, 3.5/1.7); and icterus index, 17 units. Hepatic venous catheterization was done on December 6, 1955. At that time, the icterus index was 10 units and BSP retention was 25 per cent after 30 minutes. X-ray studies on December 7, 1955, showed large esophageal varices and no demonstrable ulcer. An end-to-side portacaval anastomosis was performed on December 27, 1955; portal venous pressure fell from 350 to 140 mm. saline. Three weeks after the shunt, mental confusion, but no tremor, was first noted. The repeat catheterization was done on January 17, 1956. Laboratory studies then showed: serum proteins, 6.6 Gm. per cent (A/G, 3.4/3.2); icterus index, 15 units; and BSP retention of 30 per cent after 30 minutes. Since then he has had periodic mental confusion and a continuous mild "flapping" tremor. In January, 1957, he suffered a period of marked disorientation accompanied by a gross "flapping" tremor from which he recovered rather rapidly. There have been several subsequent one to two day episodes of marked confusion and

gross tremor. There has been no recurrence of variceal bleeding.

C. H. (#1642–082), a white man aged 46 years, had consumed alcohol in moderate excess for many years. He had suffered hematemesis in April and in December of 1955. In October, 1955, diabetes mellitus was diagnosed and treatment instituted. Another large hematemesis occurred on February 2, 1956, and he was admitted to the hospital. Hepatosplenomegaly was present and, in addition, a large tortuous abdominal collateral vein was noted leading from the umbilicus to the right epigastrium, over which a loud continuous "hum" was audible. The bleeding ceased spontaneously, and hepatic venous catheterization was performed on February 28, 1956. At this time, laboratory studies showed: serum proteins, 6.3 Gm. per cent (A/G, 4.0/2.3); icterus index, 9 units; and BSP retention of 10 per cent after 30 minutes. X-ray examination revealed large esophageal varices. An end-to-side portacaval anastomosis was done on March 19, 1956, with portal venous pressure falling from 340 to 150 mm. saline. Convalescence was complicated only by a mild transient icterus. The repeat hepatic venous catheterization was performed on April 3, 1956. Laboratory studies then revealed: serum proteins, 6.2 Gm. per cent (A/G, 3.5/2.7); icterus index, 13 units; and BSP retention of 18 per cent in 30 minutes. X-ray examination in April, 1956, showed a marked diminution in the size of the esophageal varices. Except for periodic low grade jaundice and weakness, he has remained fairly well since that time. There has been no gastrointestinal bleeding.

J. J. (#1537–328), a 43 year old white man, was admitted to the hospital on November 20, 1954, for the complaint of ankle edema for five months. There was a history of excessive alcoholic intake and two years before, while in a state prison, he was told he had cirrhosis. There was no history of jaundice, ascites, hematemesis or melena. On admission, in addition to ankle edema, hepatosplenomegaly, spider angiomata and "liver palms" were noted. Laboratory studies revealed: serum proteins, 7.2 Gm. per cent (A/G, 3.6/3.6); and BSP retention of 19 per cent after 30 minutes. X-ray examination and esophagoscopy both revealed extensive esophageal varices. Hepatic venous catheterization was done on January 19, 1955, a few hours before an end-to-side portacaval anastomosis was performed. Portal venous pressure fell from 360 to 19 mm. saline after the shunt. Liver biopsy showed portal cirrhosis. The postoperative course was uneventful, including the absence of icterus. The postoperative catheterization was done on February 9, 1955. A marked reduction in the size of the spleen was noted at this time, and laboratory studies revealed: serum proteins, 6.3 Gm. per cent (A/G, 2.7/3.6). Ankle edema recurred and has persisted to date. X-ray evamination in October, 1955, still demonstrated esophageal varices, although greatly reduced in size. The patient was confined in the State Prison until July, 1955. After his release, he began drinking and using narcotics and was seen infrequently. When last examined in December, 1956, his behavior was irrational and there was a mild "flapping" tremor of his hands. The serum proteins were 7.5 Gm. per cent (A/G, 3.1/3.4). He was again confined to prison. Detailed information could not be obtained regarding his mental state, other than to the effect that he showed evidence of mental deterioration during the two months he had spent in jail.

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